Inhibitors of Protein Kinases and Protein Phosphatases

Editors Lorenzo A. Pinna and Patricia T. W. Cohen



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Inhibitors of Protein Kinases and Protein Phosphates

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Preface

Nearly all aspects of cell life (and death) are controlled by the phosphorylation of proteins, which is catalysed by protein kinases (PKs) and reversed by protein phosphatases (PPs). The role of PKs can be likened to that of interpreters, who translate stimuli and signals into biochemical events. For this reason, PKs and PPs are themselves interlinked and highly regulated, forming complex communicative networks. Not surprisingly, therefore, the deregulation of PKs results in cell malfunction, eventually resulting in neoplastic growth and other diseases. This makes PKs attractive targets for drugs not only to combat cancer, but also for other global diseases, notably diabetes, inflammatory and infectious diseases, stroke, hypertension and Alzheimer's. Actually about half of all proto-oncogenes so far identified encode PKs, and oncogenesis frequently results from the activation and/or overexpression of PKs. For example, overexpression of the epidermal growth factor receptor tyrosine kinase is the cause of many cancers of epithelial cell origin. In other instances, however, the link of PKs with neoplasia is not so straightforward, and depends on defective interactions with cellular partners of PKs, susceptibility to particular metabolic conditions, abnormal levels of other regulatory components or the combination of several of these factors.

The attractiveness of PKs as targets is enhanced by the fact that they are enzymes, which are targetable molecules par excellence. Thus their biological activity can be turned off very easily and precisely by drugs that block the catalytic site. Virtually all PKs belong to the largest single family of enzymes, numbering over 500 and accounting for almost 2% of the proteins encoded by the human genome. They share similar catalytic domains that catalyse the transfer of phosphate from ATP to serine, threonine or tyrosine residues in key regulatory proteins. Nevertheless, the structures of the catalytic domains of PKs are sufficiently distinctive that it is possible to develop compounds that are highly selective for a particular PK. Even the highly conserved binding site for the substrate ATP is surrounded by structural elements with variable features that can be exploited for the design of specific inhibitors, and most of the PK inhibitors currently undergoing human clinical trials are of this type. Two PK inhibitors are already in clinical use for the treatment of cancers (Gleevec and Iressa), while another is the immunosuppressant of choice to prevent tissue rejection after organ transplantation (rapamycin). At least 30 other PK inhibitors are undergoing human clinical trials to treat cancers and other diseases. These have the potential to provide a significant impact on the management of epithelial cancers, such as breast and lung cancer. The approval of Gleevec for the treatment of a form of leukaemia by the FDA in May 2001 and more recently for the treatment of stomach cancers was a landmark because it is the first drug to be developed by targeting specific PKs. Moreover, its spectacular clinical effects, with minimal side effects, have had an enormous impact on the pharmaceutical and biotechnology industry. As a result, PKs have become the second most important family of drug targets, 20%–30% of all drug development programmes now being concentrated in this area. Although most PK inhibitors currently under investigation as potential drugs are ATP site-directed ligands, the field is still in its infancy, and there is tremendous potential to develop different types of drugs that target the binding sites for the protein substrates or which prevent the activation of PKs, since many of these enzymes are arranged in 'cascades' in which one PK activates or inhibits another one. Longer-term strategies would involve approaches based on gene therapy in which the mutant PK would be replaced by the wild-type enzyme.

PPs have received less attention to date as potential drug targets than PKs. The empirical discovery of an immunosuppressant drug that revolutionised organ transplantation (ciclosporin) and the subsequent recognition that it is a specific inhibitor of one PP indicates that PPs can be effective drug targets. An anticancer agent also discovered empirically (fostriecin) is now recognised to be a PP inhibitor. Other PPs, such as PTP1B, are currently under active investigations as drug targets for the treatment of diabetes and other diseases. As with PKs, known PP inhibitors at present target the active site but since many PPs are complexes with regulatory subunits, there is a potential for developing drugs that target the binding site of these regulatory subunits or their interaction with regulators. Thus the expansion of PPs as suitable drug targets may eventually follow that of PKs.

This volume of HEP highlights the tremendous pharmacological potential of PK and PP inhibitors, by providing a thorough overview of the most remarkable achievements in the field and illustrating how beneficial these studies can be for the advancement of both basic knowledge on biological regulation and deregulation and for the clinical treatment of a wide spectrum of diseases.

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Protein Kinase Inhibitors for the Treatment of Disease: The Promise and the Problems

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1 The Promise

The reversible phosphorylation of proteins, catalysed by protein kinases and phosphatases, was first identified as a regulatory device in the 1950s, and it has been established for many years that this control mechanism regulates most aspects of cell life. However, it was only in the 1990s that interest in developing inhibitors of protein kinases and phosphatases started to enter centre stage (see Cohen 2002a,b for historical reviews). The first two drugs shown to target these classes of enzyme were cyclosporin, an inhibitor of protein phosphatase 2B (PP2B, also called calcineurin) (Liu et al. 1991) and rapamycin, an inhibitor of the protein kinase mTOR (mammalian target of rapamycin) (Heitman et al. 1991), which are the immunosuppressants that have permitted the widespread use of organ transplantation. However, these drugs were developed and approved for clinical use before their mechanism of action was identified. Fasudil, an isoquinoline sulphonamide that inhibits several protein kinases with relatively low potency, such as the Rhodependent protein kinases (ROCK) (Davies et al. 2000), was developed by Hiroyoshi Hidaka in the 1980s and approved in Japan in 1995 for the treatment of cerebral vasospasm. ROCK can constrict blood vessels by inhibiting smooth muscle myosin phosphatase, but whether the clinical efficacy of fasudil results from its inhibition of ROCK, another protein kinase(s) or a completely different target, is unclear. Current information about this drug is discussed by Hidaka et al. (in Part 4).

Glivec (also called imatinib and STI-571), developed by Nick Lydon and his colleagues at Novartis, was the first drug to be developed by targeting a specific protein kinase and was approved for clinical use in the USA in 2001. It targets the protein tyrosine kinase c-Abl, which is mutated to the constitutively active BCR-Abl fusion protein in nearly all cases of chronic myelogenous leukaemia (CML). The spectacular efficacy and minimal side effects of Glivec, first highlighted by Brian Druker, resulted in the most rapid approval of a drug in FDA history and was a landmark event in this area. The development of Glivec and its implications for the future of drug discovery in this area are discussed by Fabbro et al. (in Part 4). Interestingly, Abl is not the only protein tyrosine kinase targeted by Glivec. It also inhibits the c-Kit receptor tyrosine kinase and the platelet-derived growth factor (PDGF) receptor. The c-Kit receptor is mutated to an abnormally active form in many gastrointestinal stromal tumours (GISTs) and the efficacy of Glivec for the treatment GISTs is equally impressive, resulting in its approval for this therapeutic use in 2002. The potential of Glivec to treat several types of cancer is discussed by Druker (in Part 4).

Following on from the successful launch of Glivec, Iressa a potent inhibitor of the epidermal growth factor (EGF) receptor tyrosine kinase was approved in Japan in 2002 and in the USA in 2003 for the treatment of some types of lung cancer. Developed by AstraZeneca, this drug is discussed by Wakeling (in Part 4). Drugs that inhibit the vascular endothelial-growth factor (VEGF) or fibroblast growth factor (FGF) receptor tyrosine kinases are undergoing phase III clinical trials and may be among the next protein kinase inhibitors to be approved for clinical use. VEGF and FGF play key roles in angiogenesis, and inhibitors of their receptors destroy the tumour's vascular supply. For this reason these compounds may be useful for the treatment of several types of cancer.

Compounds that inhibit protein serine/threonine kinases are also undergoing human clinical trials in a number of therapeutic areas. For example, at least four companies have inhibitors of p38 mitogen-activated protein (MAP) kinase in the clinic. These compounds suppress the production of tumour necrosis factor (TNF) and some other proinflammatory cytokines and show efficacy for the treatment of rheumatoid arthritis and other chronic inflammatory diseases. These programmes are discussed by Kumar and Blake (in Part 2). In the same section, Meijer (in Part 2) discusses inhibitors of cyclin-dependent protein kinases (CDKs), which are undergoing clinical trials as anti-cancer agents, and inhibitors of GSK3 which, although at the preclinical stage, have shown potential for the treatment of several diseases including type II diabetes (Cline et al. 2002; Ring et al. 2003) and stroke (Cross et al. 2001). Inhibitors of MAP kinase kinase 1 (MKK1, also called MEK) and RAF (product of the proto-oncogene Raf) are undergoing clinical trials as anti-cancer agents, and inhibitors of mixed lineage kinase 3 (MLK3) to prevent neurodegeneration (reviewed in Cohen 2002b). However, this is only the 'tip of the iceberg'. Over the past few years protein kinases have become the second most studied group of drug targets after G protein-coupled receptors, accounting for a quarter or more of drug discovery programmes

worldwide. The number of protein kinase inhibitors undergoing human clinical trials at the present time almost certainly exceeds 100.

The discovery that PP2B, a serine/threonine-specific protein phosphatase, was inhibited specifically by cyclosporin highlighted the potential of protein phosphatases as drug targets, and programmes to develop specific inhibitors of several of these enzymes are underway. Protein tyrosine phosphatase IB (PTP1B) appears to be one of the enzymes that dephosphorylates and inactivates the insulin receptor, because mice that do not express it are hypersensitive to insulin and maintain normal blood glucose levels at half the normal circulating of insulin (Elchebly et al. 1999). In addition, these mice do not become obese when fed a high-fat, high-carbohydrate diet. For these reasons, PTPIB is potentially an attractive target for the development of a drug to treat diabetes and/or obesity, as discussed by Cheng et al. (in Part 3). However, although interesting compounds have been developed that are relatively specific inhibitors of PTP1B, as discussed by Møller (in Part 3), no inhibitors of this enzyme appear to have entered clinical trials. CD45 is another protein tyrosine phosphatase that is potentially an attractive drug target, because it is only expressed in cells of the immune system and is essential for T cell activation. Inhibitors of CD45 therefore have the potential to be effective immunosuppressants, but may lack the side effects associated with cyclosporin and rapamycin whose targets (PP2B and TOR) are expressed in nearly all cells and tissues. This topic is discussed by Alexander (in Part 3).

A number of toxins and tumour promoters are potent inhibitors of several members of one of the major classes of protein serine/threonine phosphatases, termed the PPP subfamily. They include the marine toxins responsible for diarrhetic seafood poisoning (okadaic acid and related compounds) and the algal toxins that are a threat to water supplies (microcystins) (reviewed in MacKintosh and MacKintosh 1994). Indeed, microcystins are the most potent liver carcinogens known to man. One might therefore predict that compounds which inhibit the catalytic subunits of these protein phosphatases would frequently be oncogenic and of little use as therapeutic agents. However, as discussed by Honkanen (Part 3), both fostriecin and cantharidin, which inhibit the same protein phosphatases, are cytotoxic for tumour cells and have been tested in phase I human clinical trials as anti-cancer agents. Not surprisingly, there are a number of side effects associated with the use of these compounds, and it seems more likely that drugs will eventually be developed that disrupt the functions of protein serine/threonine phosphatases in more subtle and specific ways. For example, the ability of the serine/threonine-specific protein phosphatase 1 (PP1) to dephosphorylate many proteins is controlled by its interaction with a great variety of 'targeting' subunits that direct it to specific subcellular locations and confer unique regulatory properties upon it. The form of PP1 associated with liver glycogen, which dephosphorylates and activates glycogen synthase, comprises the catalytic subunit of PP1 complexed to a glycogen-targeting subunit G_L . The ability of the PP1- G_L complex to dephosphorylate glycogen synthase is prevented when the active form of glycogen phosphorylase (termed phosphorylase a) binds to the extreme C-terminus of G_L , providing a mechanism for inhibiting glycogen synthesis when glycogenolysis is activated and vice versa (Armstrong et al. 1998). A drug that prevented the interaction of phosphorylase a with G_L would have the potential to lower the concentration of glucose in the blood by activating glycogen synthase and so stimulating the conversion of glucose into liver glycogen.

1.1 The Problems

There are over 500 protein kinases encoded by the human genome, most of which are members of the same superfamily. This has created a plethora of potential targets that can be studied in a unified way, but has highlighted the difficulty in developing compounds that are capable of inhibiting one of these enzymes specifically. The development of Glivec has shown that inhibition of more than one protein kinase can sometimes be beneficial, allowing the same drug to have more than one therapeutic use. However, more frequently one would expect such a lack of specificity to give rise to unwanted or unacceptable side effects. The recent availability of large panels of protein kinases (e.g. Davies et al. 2000; Bain et al. 2003) has been of considerable help in assessing the specificities of protein kinase inhibitors, and it is to be expected that such panels will continue to expand and eventually include the entire repertoire of protein kinases.

Lack of specificity may also mean that the therapeutic effect of a drug is actually mediated by inhibition of another protein kinase and not by inhibition of the kinase for which it was originally developed. For example, inhibitors of the cell cycle regulator CDK2 have been developed that suppress the proliferation of tumour cells, but these compounds may actually exert their therapeutic effects by inhibiting other protein kinases, such as CDK7 and/or CDK9, which are regulators of RNA polymerase II. It is therefore unclear whether the effects of these compounds are really mediated via CDK2. In order establish that the therapeutic effect of a drug is mediated by inhibition of a particular protein kinase one needs to show that the effects of the drug disappear in cells that express a drug-resistant mutant of the protein kinase (Eyers et al. 1999). It is possible to convert protein kinases to drugresistant forms by single amino acid replacements (Brown et al. 1995; Eyers et al. 1998) so that, as for other types of drug, the development of drug resistance is a potential hazard. Mutations in Abl that make it resistant to Glivec are the cause of relapse in patients with chronic myelogenous leukaemia (Gorre et al. 2001). However, resistance to Glivec is mainly seen in patients

with the most advanced stage of this disease, where extensive genomic instability has already taken place.

Most of the protein kinase inhibitors developed thus far target the ATPbinding site and must therefore be of sufficient potency to compete with the millimolar concentrations of ATP that are present in the intracellular milieu. Clearly, it is possible to develop compounds with the requisite in vivo potency, as shown by the number of compounds undergoing human clinical trials. However, this remains a challenging problem, especially for protein kinases that bind ATP particularly tightly. Some of the most interesting protein kinase inhibitors developed thus far, including Glivec (Schindler et al. 2000) and the p38 MAP kinase inhibitor BIRB 796 (Pargellis et al. 2002), not only target the ATP-binding site, but also trigger structural changes that induce the inactive conformations of these protein kinases. Two other compounds, PD 98059 and U0126, do not target the ATP-binding site at all, but bind to the inactive conformation of MKK1, preventing it from being activated by the protein kinase Raf (Alessi et al. 1995; Davies et al. 2000). The development of more compounds that prevent one protein kinase from activating another may be a promising strategy for novel drug development in this area, since many of these enzymes are components of protein kinase 'cascades'. Another way of generating compounds that are not ATP-competitive would be to target the binding sites for protein substrates, a topic discussed by Lawrence (in Part 1).

There are about 150 protein phosphatase catalytic subunits encoded by the human genome, and they fall into three main superfamilies. The generation of compounds that discriminate between different protein phosphatases is therefore also a challenging one. However, in contrast to protein kinases, the option of targeting an ATP binding pocket does not exist. Moreover, the protein substrate-binding cleft can be very polar, as in the case of PTP1B (Kellie 2003). This has made it difficult to develop compounds that combine high potency with cell permeability. The only protein phosphatase inhibitor that has advanced to human clinical trials, cyclosporin, inhibits PP2B in an unusual way; it binds to the protein cyclophilin, and the cyclosporin-cyclophilin complex then inhibits the protein phosphatase (Liu et al. 1991). As discussed earlier, it seems more likely that the future of drug discovery in this area may lie in targeting the regulatory subunits of serine/threoninespecific protein phosphatases.

Finally, it is important to mention that inhibitors of protein kinases are not only becoming important for the treatment of disease, but also as reagents for the study of cell signalling. The huge number of citations garnered by the publications that have introduced these compounds to the scientific community are a reflection of the widespread need for these compounds by the scientific community. For example, I was surprised to learn from the Institute for Scientific Information that the paper we published in 1995 with David Dudley and Alan Saltiel at Parke Davis on the mechanism of action of PD 98059 (Alessi et al. 1995) was the UK's most frequently cited original research paper over the past 10 years in the fields of biology and biochemistry, while our publication with Peter Young and John Lee at SmithKline Beecham on the specificity of SB 203580 (Cuenda et al. 1995), a prototypic p38 MAP kinase inhibitor, was the UK's sixth most cited original research paper over this period. Although many compounds are advertised for sale as 'specific protein kinase inhibitors', in practice many have turned out to inhibit so many protein kinases that conclusions drawn from their use are likely to be erroneous (Davies et al. 2000; Bain et al. 2003). The number of really useful protein kinase inhibitors that are available commercially is still rather limited, but the number will increase considerably over the next few years. I believe that pharmaceutical companies have much to gain from the discoveries that will be made by exploiting these compounds, and it is to be hoped that many more will be released for general use in the future.

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Part I General Aspects of PKs Inhibition

New Design Strategies for Ligands That Target Protein Kinase-Mediated Protein–Protein Interactions

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Abstract Protein–protein interactions serve as the molecular engine that drives the formation and disassembly of intracellular signaling pathways. Antagonists of these interactions could play key roles as both biological reagents and therapeutic compounds. However, much of the early work in this area with peptides revealed that these species, in general, bind with modest affinity to their protein targets. In addition, when these studies first commenced nearly 20 years ago, the technology for the intracellular delivery of peptides and modified analogs thereof was rudimentary. In the intervening years, not only has this technology dramatically improved, but the global role that protein–protein interactions play in transducing intracellular signals has become simply too obvious to ignore. With the introduction of combinatorial library methods, it is now a simple matter to identify consensus sequences recognized by protein interaction domains. An array of strategies has now been developed to transform these otherwise modest binding consensus sequences into high-affinity ligands. These strategies include the design of high-affinity replacements for key amino acid residues in consensus peptides, the construction of multidomain-binding peptides, and the structural modification of consensus sequence peptides. In several of these instances, unprecedented affinity (<nM) and selectivity (>1,000-fold versus closely related protein targets) have been achieved.

Keywords Signal transduction \cdot Antagonists of protein–protein interactions \cdot Peptidebased inhibitors \cdot Protein kinases and phosphatases \cdot Combinatorial libraries \cdot Amino acid analogs \cdot Bivalent inhibitors \cdot Structurally modified peptides

1 Introduction

Protein-protein interactions serve as the adhesive that drives the assembly of signaling pathways. However, this adhesive is transient in nature. Once the cell has acknowledged the environmental stimulus, signaling pathways must rapidly disassemble to restore the cell to its resting state. At first glance, agents that selectively target key protein-protein interactions would appear to serve as ideal inhibitors of cell signaling as well as potential therapeutics. First, protein-protein interactions are typically exemplified by welldefined consensus sequences, which can often be reasonably selective for a given protein-protein pair. Consequently, the preparation of inhibitors of protein-protein interactions appears, at least on paper, to be reasonably straightforward since, the acquisition of preferred consensus sequences employs simple and well-defined methods. Second, the intracellular levels of protein-protein-binding partners rarely surpass low micromolar amounts, thereby rendering competition with endogenous substrates relatively unimportant. In spite of these apparent advantages, the overwhelming majority of reported protein kinase inhibitors target the ATP-binding site, a region common to all protein kinases, non-protein kinases, and many other ATP-binding proteins. Furthermore, the intracellular concentration of ATP (~1-10 mM) is much larger than its K_m (serine/threonine kinases ~1–10 μ M; tyrosine kinases ~20–50 μ M), which all but assures that the ATPbinding site will be saturated with ATP. The consequence of the latter is that inhibitors that target the ATP-binding site must be present at intracellular concentrations that significantly exceed their in vitro-determined K_i values. Finally, the acquisition of ATP analogs that specifically target individual protein kinases requires the initial screening of a large starting library of potential inhibitor candidates. This is then followed by a substantial synthetic effort that involves the preparation of secondary and tertiary libraries based on initially identified leads. The notion of disrupting signaling pathways via antagonists of protein-protein interactions has been unpopular for a number of reasons, including issues related to potency, intracellular stability and uptake, and general bioavailability (i.e., with respect to therapeutics). However, recent advances in various delivery technologies coupled with our increasing understanding of the widespread participation of protein-binding domains in signaling, has led to a renewed interest in the development of anti-signaling agents that disrupt intracellular protein-protein interactions.

Given the long-dormant state of this field, which is characterized by a recent reawakening, a broad overview of the general area of protein kinasemediated protein-protein interactions and their corresponding antagonists is provided. This includes a summary of the methods employed to obtain consensus sequence information, a general synopsis of protein-binding domains, and finally a description of antagonists of protein-protein interactions as well as emerging strategies to acquire ever more potent and selective inhibitory agents.

2 Identification of Consensus Sequences

2.1 Degradation of Protein Ligands

Amino acid recognition sequences that drive protein-protein interactions were initially identified via partial digestion of one of the protein-binding partners. Fragments that were determined to retain binding potency were then sequenced. Further refinement of the amino acid recognition sequence could then be explored via the preparation of synthetic peptides. This strategy is best exemplified by the work described in the 1980s on the potent "heat-stable" inhibitor of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) known as PKI (protein kinase inhibitor). Krebs, Walsh, and their colleagues (Scott et al. 1985a,b; Cheng et al. 1986; Scott et al. 1986; Van Patten et al. 1986; Glass et al. 1989) identified a series of peptides that serve as extraordinarily potent inhibitors (Ki<50 nM) of PKA. Protease digestion of the isolated protein furnished a 20-mer peptide that acts as a competitive inhibitor versus peptide substrate with a K_i in the subnanomolar range. These investigators demonstrated that the sequence Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile is the active site-directed component of PKI, where the Ala residue is positioned at the site normally reserved for the phosphorylatable serine. Indeed, subsequent studies demonstrated that insertion of serine in active site-directed sequences derived from PKI generates powerful peptide substrates (Mitchell et al. 1995). However, the new library-based methods introduced in the 1990s have largely supplanted the biochemical approaches for identifying amino acid sequences recognized by protein interaction domains. The new methodologies are not only significantly less labor intensive than their classical counterparts, but are also able to bypass the need for large quantities of both binding partners (for digestion and sequencing purposes).

2.2 Synthetic Peptide Libraries

A large number of different library strategies using synthetic peptides have been described. These approaches include one-bead/one-peptide libraries (Wu et al. 1994), solution mixtures of peptides (Songyang et al. 1994), onewell/one-peptide strategies (Lee and Lawrence 1999), peptides on chips (Houseman et al. 2002), and even proteins on chips (Zhu et al. 2000). A detailed description of the vast array of peptide library strategies now available is well beyond the scope of this review. However, all of these methods offer a rapid means to quickly identify preferred amino acid sequences in what is typically a single experiment. Peptide-based libraries also permit the use of amino acid derivatives beyond the standard genetically encoded residues (e.g., post-translationally modified residues such as phosphoTyr, hydroxyPro, etc.). In addition, many of the methods not only identify a preferred consensus sequence, but also often furnish an assessment of the range of residues permitted at a given position on the peptide ligand.

Each of the peptide library strategies enjoys certain advantages while enduring specific disadvantages:

- 1. One-bead/one-peptide libraries are extremely easy to prepare via split-andpool synthesis (Lam et al. 2003). However, these libraries are commonly composed of a mixture of millions of beads, with each bead possessing a unique peptide sequence. Consequently, a screening method must be devised so that the bead containing the tightest binding ligand can be readily identified. Possibilities include the use of a target protein that contains an appended fluorophore or is conjugated to an enzymatic reporter. Beads can also be identified via the introduction of radioactivity (i.e., the use of $[\gamma$ -³²P]ATP). Once leads have been identified, the beads are isolated and the bound peptides identified by microsequencing. Given the heavy reliance upon the latter, the use of uncommon hypermodified residues is severely restricted.
- 2. Soluble peptide library mixtures have also been utilized to identify consensus sequences (Songyang and Cantley 1998). These libraries are prepared by treating the growing peptide chain with a mixture of the standard amino acid derivatives. The actual ratio of the amino acids introduced during the coupling reaction is based upon the relative coupling efficiencies of the individual residues. Consequently, a particular residue that couples sluggishly (e.g., Arg) is present at a greater relative ratio than one that couples readily (e.g., Gly). Following completion of the synthesis, the peptide mixture is cleaved from the resin and subsequently employed for consensus sequence identification. The latter is achieved by selective enrichment of the binding sequence, often using an affinity column. For example, protein kinase-catalyzed phosphorylation of the mixture is allowed to proceed until a small fraction (<1%) of the total peptide is phosphorylated. The phosphopeptide

mixture is subsequently isolated and sequenced as a mixture. Each position on the peptide is not identified as a single residue, but rather as the relative abundance of all the amino acid residues at a particular site. The residue present in the largest amount at a given position is taken as the one most favored at that site. However, since a peptide mixture, as opposed to a single peptide, is sequenced, this strategy does not yield sequences of unique peptides but merely determines the preferences for particular residues at specific positions. An inherent assumption of this method is that selection at each position is independent of the adjacent amino acids. Consequently, this technique ignores the possibility that two or more residues can act in a synergistic fashion to promote target protein affinity.

- 3. The one-well/one-peptide approach ("parallel synthesis") (Granier 2002) employs pure peptides that are spatially segregated from one another (Lee and Lawrence 1999). This technique has the advantage that the sequence of each peptide in each well is verified in advance. Furthermore, a wide assortment of hypermodified amino acid residues can be employed, since the synthesis history of each peptide in each well in known. An obvious disadvantage is that the size of these libraries, by necessity, is much smaller than those described in points 1 and 2 above. Variations that employ spatially segregated mixtures ("positional scanning") have been reported that address this concern (Houghten et al. 1996).
- 4. Peptide chips represent the solid phase version of the method described in 3 (Houseman et al. 2002). The added advantage of this system is the higher spatial density, and therefore smaller chip size [membranes have been employed in this technique as well (Frank 2002)]. However, the increased spatial density of the individual peptide "colonies" can come at a cost. Although methods that employ fluorescence detection of target protein binding will work well in this system, other common methods, such as those that utilize radioactivity, cannot be applied to ultra high-density chips.

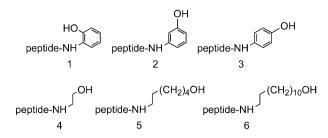
2.3 Phage Display

Phage display is a genetically encoded peptide library strategy (Scott and Smith 1990; Smith and Scott 1993). In brief, peptides are displayed on the capsid protein of filamentous phage. Each virion particle displays a unique peptide sequence on its surface. Millions of phage clones are exposed to the protein target of interest and the affinity purified particles then amplified in *Escherichia coli*. Subsequent rounds of selection furnish a few "lead" clones, from which the displayed sequences can be determined via sequencing of the viral DNA coding region. Phage display has been used to acquire peptide ligand sequences for a wide variety of protein interaction domains (Smothers et al. 2002). The obvious limitation here is that the genetic basis for this method restricts the range of amino acids to the 20 standard residues.

3 The Protein-Binding Domains of Protein Kinases

Protein kinases are, first and foremost, catalysts that promote the transfer of a phosphoryl group from ATP to the acceptor hydroxyl moiety of serine, threonine, and/or tyrosine. The serine, threonine, and tyrosine residues must be embedded within the proper amino acid sequence in order to be recognized by a given protein kinase, a fact exemplified by the large number of synthetic peptide-based substrates that have been devised for scores of protein kinases. Of all the protein-binding domains contained within protein kinases, the active site region displays the greatest diversity in terms of sequence recognition. However, since all members of the protein kinase family utilize the same phosphoryl donor (ATP) and acceptors (serine, threonine, tyrosine), it is perhaps not too surprising that the conformation of the active site region is remarkably well-conserved (Johnson et al. 1998; Huse and Kurivan 2002). The "protein kinase fold" is composed of two separate lobes, commonly designated as the N- and C-terminal lobes. The former is the smaller of the two and is composed of five antiparallel β -strands and a single α -helix. The larger C-terminal lobe is primarily α -helical in structure. ATP resides in a cleft that lies at the interface between the N- and C-terminal lobes. By contrast, the peptide/protein phosphoryl acceptor is primarily associated with the C-terminal lobe. The catalytic domain of protein kinases can assume active and inactive conformational states. The lobes in the former migrate toward one another, thereby closing the active site and promoting catalysis.

Protein kinases are commonly differentiated on the basis of their preferred phosphoryl acceptor group on the protein substrate: either the aliphatic hydroxyl moieties of serine and threonine ("serine/threonine protein kinases") or the aromatic phenol of the tyrosine residue ("tyrosine protein kinases"). A few protein kinases display the property of "dual specificity" in terms of their ability to recognize and phosphorylate both aliphatic and aromatic alcohols on peptides or proteins in vitro (fewer still display this property in living cells) (Dhanasekaran and Premkumar Reddy 1998; Marin et al. 1999). However, in a very strict sense, the segregation of protein kinases into these separate camps most likely has less to do with the protein kinases themselves and more to do with the fact that the genetic code is limited to only 20 different amino acids. For example, PKA, a well-known serine/threonine-specific protein kinase, phosphorylates appropriately designed aromatic alcohols (e.g., 1-3) (Lee et al. 1994).



Furthermore, Src, an equally well-established tyrosine-specific protein kinase, phosphorylates aliphatic alcohols (e.g., **4–6**) (Lee et al. 1995a,b). Obviously, from the biological point of view, compounds **1–6** are mere curiosities. However, in terms of designing sensors, substrates, and antagonists of protein kinase-mediated protein-protein interactions, derivatives **1–6** are an important reminder that, unlike cells, chemists are not limited to the standard 20 amino acids fixed by the genetic code. Indeed, early work with consensus sequence peptides *containing conventional amino acids* is, in large part, responsible for the prevailing notion that inhibiting signaling pathways via disruption of protein-protein interactions is a strategy doomed to failure.

In addition to the "active site specificity" (i.e., serine/threonine versus tyrosine) of protein kinases, these enzymes display a preference for the amino acid sequence that encompasses the phosphorylatable residue (the "sequence specificity"). Compilations of sequences phosphorylated by protein kinases are available and these will not be recapitulated here (Pinna and Ruzzene 1996). However, certain trends are apparent:

- 1. The overwhelming majority of protein kinases will also phosphorylate simple peptides, thereby rendering the in vitro assay of these enzymes fairly straightforward. In addition, this demonstrates that the protein-binding region of these enzymes is sufficiently structurally well established to recognize substrates on its own (i.e., large intact protein substrates are not required for the protein kinase to assume an active state). However, the notion that the protein-binding region is the only site on the protein kinase that is responsible for substrate specificity is decidedly untrue.
- 2. Absolute protein kinase specificity is not encoded within the substratebinding site. In other words, the consensus sequence surrounding the phosphorylatable residue, although an important parameter of protein kinase recognition, is not the sole determinant of specificity. For example, the cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and PKA, members of the same protein kinase subfamily, display largely overlapping specificities with respect to simple peptide substrates (Mitchell et al. 1995; Wood et al. 1996). Certain protein kinase C (PKC) isoforms likewise phosphorylate the same peptides as PKA and PKG; however, the sequence preference of PKC is broad enough that PKC peptide substrates have

been devised that are recognized by neither PKA nor PKG (Yan et al. 2000). In short, it is unlikely that a peptide composed of only conventional amino acids will serve as an absolutely specific substrate for any given protein kinase. Indeed, Cohen and his colleagues have used this notion to generate a small set of peptides that serve as general substrates for more than three dozen different protein kinases (Ross et al. 2002).

3. Consensus sequence-containing active site-directed peptides are generally poor inhibitors. This fact is responsible for much of the common belief that targeting the substrate recognition site in particular, and protein-protein interactions in general, is an untenable strategy. Perhaps the bestknown example is kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, which serves as an excellent substrate for PKA. The $K_{\rm m}$ for this peptide is less than 20 µM, a value that was (incorrectly) taken as a reflection of the binding constant of the peptide. However, the corresponding nonphosphorylatable peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly, is an exceedingly poor PKA inhibitor (K_i =320 μ M) (Whitehouse et al. 1983). Much of the early discussion concerning the ineffectiveness of the Ala-substituted peptide centered on the possibility that the serine hydroxyl group (missing in the inhibitor) promotes binding affinity by two orders of magnitude. However, subsequent detailed enzymological studies revealed that the K_m value is a complex parameter that is dependent upon more than just the microscopic rate constants that control the active site association and dissociation of peptide substrate (Adams and Taylor 1992). In an analogous vein, poor inhibitors of tyrosine kinases (in which the phosphorylatable tyrosine residue was replaced with a phenylalanine) have been noted. Nevertheless, a few exceptions to the "rule" that active site-directed peptides serve as poor inhibitors are known. A naturally occurring "heat stable" protein-based inhibitor (PKI) of PKA is a powerful inhibitor ($K_i < 1$ nM) (Whitehouse and Walsh 1983). A peptide fragment of PKI, most notably Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Leu-Gly-amide (where the Ala represents the site that would be reserved for serine) was reported to have a K_i of 36 nM (Glass et al. 1989). As an aside, the K_i for this peptide was originally determined under conditions of low salt; when the inhibitory potency of this peptide was subsequently reexamined several years later under more physiologically conditions, the K_i was found to be 500 nM (Wood et al. 1998). Nevertheless, the latter value does suggest that it is feasible to devise reasonably potent inhibitors based on standard amino acid residues alone. Indeed, perhaps the most outstanding example of this is the extraordinarily potent 24 amino acid-containing PKG selective inhibitor Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Typ-Lys-Lys-Leu-Arg-Lys-Lys-Lys-Lys-His (Dostmann et al. 2000). This peptide is likely engaged in interactions beyond the immediate vicinity of the active site. Indeed, there appears to be a general consensus of opinion that, unlike targeting the ATP-binding site, effective inhibitors of protein-protein interactions must coordinate to a relatively large surface area.

In addition to the active site, there are several other protein interaction domains that are commonly affixed to protein kinases. These include the SH2 and SH3 domains, which are prevalent among the tyrosine protein kinases. PDZ, LIM, WW, PTB, and others are found in many protein kinases and/or in the adaptor proteins that help to transduce the activity of these enzymes. In addition, protein kinases themselves serve as ligands for protein interaction domains present on anchoring proteins. Indeed, peptide-derived inhibitors that bind to these anchoring proteins and thereby block protein kinase docking, have been described (Csukai and Mochly-Rosen 1999). The primary focus of this chapter is on the acquisition of active sitedirected peptide-based inhibitors and the emerging strategies to acquire ever more potent and selective agents.

4 Strategies for the Acquisition of Potent and Selective Peptide-Based Inhibitors of Protein Kinases

Although a few exceptions are known, in general, conventional peptides display modest affinities and poor selectivities for the protein interaction domains contained within protein kinases. Biological systems appear to have little need for high-affinity ligands for active sites, SH2, SH3, LIM, PDZ, and other protein-interaction domains due to the transient nature of signaling pathways. However, it is abundantly clear that biological systems have mastered the issue of selectivity. Selective expression of only certain protein kinases in specific cell types, or at precise intervals during the lifetime of the cell, offers one means to navigate the tricky waters of intracellular selectivity. Spatial segregation of protein kinases to specific intracellular sites represents another means by which selectivity can be achieved. Finally, given the comparatively large size of these proteins, and their correspondingly well-defined structures, selectivity may simply be attained via a highly precise three-dimensional choreography of interactions between binding partners. Consequently, the design of potent and selective artificial antagonists of protein-protein interactions represents a significant challenge, albeit an exciting one. The primary advantage enjoyed by the chemist is that he or she is not restricted to the 20 standard amino acids designated by the genetic code.

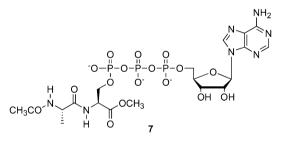
The acquisition of agents that target protein-protein interaction sites has the potential to be relatively straightforward. Consensus sequences are easy to identify. However, it is necessary to develop the tools and/or strategies that can convert peptides containing these sequences into agents that recapitulate the high selectivities observed in biochemical pathways while significantly surpassing the affinities that intracellular binding partners display for one another. The tools and strategies to achieve the twin goals

4.1 Mimetics of Key Residues in Consensus Sequence Peptides

4.1.1 Serine Analogs

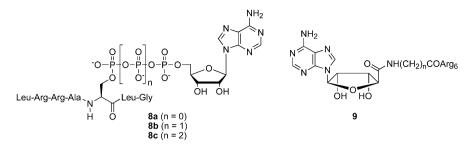
As noted above, one of the first indications that the acquisition of effective peptide-based inhibitors for protein kinases might be problematic was the replacement of the phosphorylatable serine residue in the PKA substrate kemptide with an alanine to create a dead-end inhibitor. The latter proved to be an unexpectedly weak inhibitory agent (K_i >300 µM versus the K_m for kemptide <20 µM). One of the explanations offered for the low affinity, namely loss of the hydroxyl serine side chain as a potential hydrogen bond donor, was subsequently shown to be incorrect. However, this notion does suggest that there may be ways to improve upon the use of alanine as a non-phosphorylatable replacement for serine.

Coward and his colleagues were the first to suggest that the phosphorylatable residue in an active site-directed peptide could be substituted with an analog that is able to also engage the ATP-binding site (i.e., a bisubstrate inhibitor) (Lashmet et al. 1983).



Although the ATP- γ -Ala-Ser ester 7 does not possess the requisite peptide framework for it to serve as a protein kinase inhibitor, it is a model of the type of compounds that were eventually prepared more than a decade later.

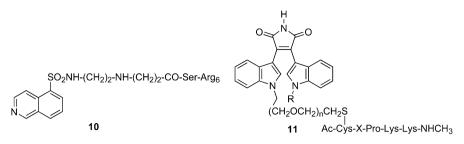
Gibson and his colleagues were the first to report the synthesis of adenosine phosphopeptides in a solid phase format (Medzihradszky et al. 1994).



These investigators described the preparation and characterization of several analogs (8) of kemptide. Although the IC₅₀ values of 8a, 8b, and 8c (935 μ M, 226 μ M, and 68 μ M, respectively) are modest, these inhibitors are significantly more effective than the simple Ala-containing analog Leu-Arg-Arg-Ala-Ala-Leu-Glu. The authors found that 8 displays a competitive inhibition pattern versus variable ATP, but such a pattern was not observed with respect to variable peptide substrate. One might expect that a bisubstrate analog would exhibit competitive patterns versus both ATP and phosphorylatable peptide. However, the absence of double competitive behavior does not necessarily rule out the two-site binding model. Strictly speaking, competitive behavior is observed for an inhibitor only if that inhibitor and the corresponding substrate bind in a mutually exclusive fashion to the same enzyme form. PKA is known to exhibit a primarily ordered mechanism with ATP binding first (Whitehouse et al. 1983). Consequently, one would expect compound 8 and ATP to associate with the same enzyme form, namely the free enzyme, and thereby exhibit competitive behavior. By contrast, given the nature of the ordered mechanism, peptide substrate preferentially coordinates to the enzyme-ATP complex, which would thereby rule out a competitive pattern with 8.

Recently Uri and his colleagues have described a series of bisubstrate analogs that dispenses with the phosphoric anhydride portion of the ATP moiety (Loog et al. 1999). These investigators employed an adenosine-5'-carboxylic acid derivative as the ATP mimic which, using a variety of linkers, was appended to the N-terminus of an arginine rich peptide (9). The most effective analogs displayed IC_{50} values of between 100 and 300 nM for PKA and PKC. These bisubstrate analogs have been used to affinity-purify protein kinases (Loog et al. 2000). In addition, membrane-permeable fluorophore-labeled bisubstrate derivatives have been prepared (Uri et al. 2002; Viht et al. 2003).

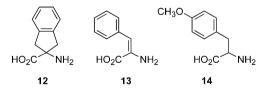
In a departure from the ATP-based bisubstrate strategy, Sergheraert and colleagues designed (ATP mimics)-linker-substrate analogs (Ricouart et al. 1991). Isoquinoline and naphthalene sulfonic acid derivatives served as ATP replacements. The most potent of the several derivatives prepared was compound **10**.



The latter exhibits a 25-fold selectivity in favor of PKA (K_i =4 nM) versus PKC (K_i =100 nM). The inhibitory potency of this derivative is impressive when one considers the fact that the ATP mimic alone is a nearly three orders of magnitude poorer inhibitor than 10. However, since the ATP analog is appended off the N-terminus of the peptide, an unanswered question is the nature of the requisite structural requirements to replace a serine moiety that is contained within the interior of a consensus sequence. Finally, 10 acts as a competitive inhibitor versus variable ATP, but is not competitive with respect to variable peptide substrate. Sasaki, Maeda, and their coworkers likewise utilized an ATP analog (a bisindolylmaleimide) to prepare a series of bisubstrate inhibitors 11 that are designed to target the cyclin-dependent protein kinase, cdc2 (Sasaki et al. 1998). The best inhibitors display IC₅₀ values in the low micromolar range (where X=no amino acid). However, when X=Ser, the inhibitory potency is reduced by two orders of magnitude.

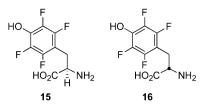
4.1.2 Tyrosine Analogs

In an analogous vein to serine/threonine protein kinases, peptide-based inhibitors of tyrosine kinases were initially prepared by substituting the phosphorylatable tyrosine with the nonphosphorylatable phenylalanine. In general, the phenylalanine-for-tyrosine replacement generates exceedingly poor inhibitory agents (K_i >1 mM).



Several peptides containing tyrosine analogs (12–14) were reported in the 1980s, but these derivatives proved to be ineffective as inhibitors (Wong and Goldberg 1984; Shoelson et al. 1989).

Graves and his colleagues described the first example of an effective peptide-based tyrosine kinase inhibitor (Yuan et al. 1990).



The inhibitory agent, which contains the tetrafluorotyrosine moiety 15, targets the insulin receptor with a K_i of 4 μ M. The rationale for the use of the fluorinated tyrosine analog was based on the presumed mechanism of catalysis. These investigators reasoned that an active site base partially removes the aromatic hydroxyl proton during the transition state of the enzyme-catalyzed phosphoryl transfer reaction from ATP to the acceptor phenol. Presumably, the enzyme stabilizes this partial-negative charge on the phenol/phenoxide during the transition state, which suggests that a tyrosine analog that is negatively charged might be well accommodated within the active site. The four fluorine substituents not only lower the pK_a of the phenol, thereby promoting ionization to the phenoxide at physiological pH, but in addition they render the phenoxide less nucleophilic than its natural counterpart. These investigators also prepared the corresponding d-analog 16, which also displays promising inhibitory activity (K_i =20 μ M). Interestingly, although both 15 and 16 serve as competitive inhibitors versus variable peptide substrate, the l-analog directly competes with ATP as well, whereas the d-derivative does not.

Subsequent work by Fry and his colleagues at Parke-Davis confirmed the usefulness of the tetrafluorotyrosine moiety as a nonphosphorylatable analog, in this case for peptides that target the epidermal growth factor receptor (EGFR) (Fry et al. 1994). The phenylalanine-containing "parent" peptide acetyl-Leu-Ala-Glu-Glu-Ser-Ala-Phe-Glu-Glu displays a K_i of 150 µM, whereas the corresponding l- and d-tetrafluorotyrosine-containing derivatives exhibit relative inhibitory enhancements of threefold and eightfold, respective-ly. The Parke-Davis group also prepared peptides that contained other l-ty-rosine analogs, including 3-fluorotyrosine, 3-iodotyrosine, and d-tyrosine, but all of these derivatives were ineffective EGFR inhibitors. Curiously, 3-io-dotyrosine was subsequently found to serve as an excellent tyrosine replacement in a cyclic peptide targeting Src (Alfaro-Lopez et al. 1998).

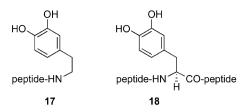
Walsh, Cole, and their colleagues also examined the use of tetrafluorotyrosine as a tyrosine replacement in a C-terminal Src kinase (CSK)-targeted peptide (Cole et al. 1995; Kim and Cole 1998). However, in this case, the peptide serves as a substrate, rather than as an inhibitor, for CSK. These results suggest that the applicability of tetrafluorotyrosine as a nonphosphorylatable tyrosine replacement is kinase-dependent.

Lam and his collaborators have prepared a series of active site-directed peptides that target the Src tyrosine protein kinase (Lou et al. 1997). These

investigators employed both d- and l-napthylalanine (Nal) derivatives in place of the phosphorylatable <u>tyrosine</u> moiety in the sequence Gly-Ile-<u>Tyr</u>-Trp-His-His-Tyr. The corresponding phenylalanine derivative was not prepared; however, the d-Tyr was, which gives a measure of the inherent affinity of the peptide for Src. The IC₅₀ for Gly-Ile-d-<u>Tyr</u>-Trp-His-His is 50 μ M, which indicates that the peptide framework is, comparatively speaking, a remarkably effective peptide-based inhibitor. The corresponding Gly-Ile-Nal-Trp-His-His derivative exhibits only a twofold improvement in IC₅₀ relative the d-Tyr analog. However, the doubly substituted Gly-Ile-Nal-Trp-His-His-Nal exhibits an IC₅₀ of 4 μ M, suggesting that the C-terminal Nal is able to access sites outside of the immediate active site region. Interestingly, one of the less effective inhibitors Gly-Ile-Nal-Trp-His-His-Tyr (IC₅₀=27 μ M) proved to be remarkably selective for Src versus other closely related members of the Src kinase family (Lyn and Lck; IC₅₀>1 mM).

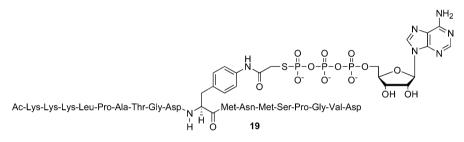
One of the difficulties associated with the acquisition of nonphosphorylatable tyrosine surrogates is their synthesis, which typically resorts to the use of achiral starting material. Following a resolution step, the analogs must then be appropriately protected for use in solid phase peptide synthesis. Some of these difficulties have been circumvented by Kim and Cole, who employed the enzyme tyrosine phenol lyase to prepare gram quantities of an assortment of fluorinated tyrosine analogs (Kim and Cole 1998). The Lawrence group has developed a library-driven strategy, which allows one to prepare and subsequently screen a wide assortment of commercially available aryl-containing amines as peptide-based nonphosphorylatable tyrosine analogs (Niu and Lawrence 1997a,b). In spite of the fact that these are peptide derivatives, issues related to synthesis, resolution, and protection of these tyrosine substitutes are all bypassed.

Although the most common protein kinase peptide substrates possess a phosphorylatable residue embedded within the interior of the peptide, protein kinases will also phosphorylate peptides containing tyrosine, serine, and threonine moieties appended off the N- or C-terminus of these substrates. For example, Src catalyzes the phosphorylation of Arg-Arg-Arg-Arg-Arg-Leu-Glu-Glu-Leu-Leu-Tyr-amide (the arginine residues are present for assay purposes, not enzyme recognition). C- and N-terminal residues can be readily appended onto the active site-directed peptide after solid phase peptide synthesis. This allows one to employ potential tyrosine analogs that are not protected, possess functionality that might not survive the harsh conditions of peptide synthesis, and even lack the standard α -stereocenter. The synthetic strategy utilizes a solid phase peptide synthesis support (Kaiser's oxime resin) that allows the tyrosine analog to be attached to the synthesized peptide in a fashion that simultaneously promotes cleavage from the resin (Kaiser et al. 1989). For example, a wide assortment of phenylethylamine derivatives was attached to the C-terminus of a Src active site-directed peptide (Niu and Lawrence 1997a,b).



These were screened for inhibitory potency, and the lead analog was identified as the dopamine derivative 17 (which, in spite of a *p*-substituted aromatic alcohol, does not serve as a substrate). A peptide containing the amino acid analog of dopamine, l-dopa (18), was subsequently synthesized and shown to display an inhibitory potency (K=16 µM) that exceeds the parent phenylalanine-containing peptide by 60-fold.

Cole and his colleagues have reported a high-affinity bisubstrate analog for the insulin receptor protein kinase (IRK) (Parang et al. 2001).



Compound 19 was designed based upon a dissociative mechanism for phosphoryl transfer in the IRK active site. The authors reasoned that the approximately 5 Å that separates the aromatic amine nitrogen from the γ -thiophosphate phosphorous roughly recapitulates the distance between acceptor and donor in a metaphosphate-like dissociative mechanism. Unlike the bisubstrate analogs reported to date for the serine/threonine-protein kinases, compound 19 serves as a competitive inhibitor versus both variable ATP and peptide substrate. The K_i of 19 is 370 nM, which corresponds to a binding energy that is roughly equal to the sum of the ATP- and protein-binding site portions of the inhibitor. As one might expect for an inhibitor that contains a peptide sequence targeting IRK, compound 19 is ineffective versus CSK ($K_i \sim 40 \mu$ M). These investigators also obtained the crystal structure of the inhibitor bound to the tyrosine kinase domain of IRK. The latter confirmed that the inhibitor is bound in a bisubstrate-like mode with the expected distance between the anilino nitrogen and the γ -phosphorous. In addition, the anilino nitrogen is engaged in a hydrogen bond to a key active site Asp residue.

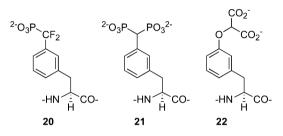
Budde, McMurray, and their collaborators reported an N-myristoylated peptide, myr-Glu-Phe-Leu-Tyr-Gly-Val-Phe-Asp-amide, that serves as an apparent bisubstrate analog for Src (Ramdas et al. 1999). Surprisingly, the cor-

responding peptide with a free N-terminus is a Src substrate. However, upon acylation the substrate is converted into an inhibitor with the caveat that the acyl group must be lauryl ($C_{11}H_{23}CO$ -) or longer. Clearly, the unexpected structure/activity relationship of this inhibitory species places it in an unusual category in that there is no obvious consolidated nonphosphorylatable tyrosine mimetic present in the peptide framework. Nonetheless, the fatty acyl-peptide serves as a competitive inhibitor with respect to both variable ATP and peptide substrate (poly Glu₄Tyr), thereby rendering it, like compound **19**, a bisubstrate analog. The nonacylated peptide itself blocks phosphorylation of poly Glu₄Tyr with a K_i of 260 μ M via a competitive pattern. Myristic acid also serves as an inhibitor of the Src-catalyzed phosphorylation of poly Glu₄Tyr, but in this instance the fatty acid competes with ATP (K_i =35 μ M).

The conjoined myr-Glu-Phe-Leu-Tyr-Gly-Val-Phe-Asp-amide exhibits bisubstrate inhibition with K_i values of 3 μ M (variable Glu₄Tyr) and 6 μ M (variable ATP). Consequently, the fatty acyl-peptide is unable to serve as a Src substrate because ATP is unable to bind to the active site in the presence of the myristyl group. Unfortunately, this inhibitory species does not display selectivity against other protein kinases (CSK, PKA, and the FGF receptor). However, it may ultimately be possible to enhance either selectivity or potency by placing the fatty acid moiety at different sites along the peptide chain to minimize the distance between the site of phosphorylation (i.e., the Tyr residue) and the ATP-binding site. These results stand in interesting contrast to earlier work described by Ward and O'Brian (O'Brian et al. 1990; O'Brian et al. 1991; Ward and O'Brian 1993). PKC peptide substrates, upon N-myristoylation, are converted into inhibitors with IC₅₀ values of between 3-10 µM, depending upon the amino acid sequence. However, unlike the myristoylated peptides that inhibit Src, Ward and O'Brian's PKC inhibitors do not display a competitive pattern versus variable ATP, nor do they serve as competitive inhibitors versus peptide substrate. The authors conclude that their myristoylated peptides bind to a different enzyme form (i.e., the free enzyme) than the peptide substrate (i.e., the enzyme-ATP complex). This accounts for the noncompetitive inhibition pattern versus peptide substrate. In addition, Ward, O'Brian, and their colleagues have suggested that the inhibitory effect conferred by the myristoyl appendage is due, at least in part, to its interaction with the phosphatidylserine cofactor of PKC (O'Brian et al. 1990). However, more recent studies suggest that a myristyl-binding region is located in close proximity to the peptide-binding region of the active site (Zaliani et al. 1998). Consequently, it appears likely that the inhibitory behavior of myristoylated peptides toward PKC is at least partly due to the presence of a near active site hydrophobic region that is able to accommodate the lipophilic fatty acid moiety.

4.1.3 Phosphotyrosine Analogs

SH2 and PTB domains, as well as protein tyrosine phosphatases, recognize sequences that encompass phosphotyrosine. However, since the latter is hydrolyzable, peptides that contain this residue serve only as transient antagonists of protein-protein interactions. A number of nonhydrolyzable phosphotyrosine mimetics have been described, in large part due to the effort of Burke and his collaborators at the NIH. Since phosphotyrosine analogs have been the topic of recent reviews, only a cursory discussion of their current status will be presented (Burke et al. 2001; Burke and Lee 2003).



Perhaps the most extensively employed of all phosphotyrosine analogs is the difluorophosphonate derivative 20. The synthesis of this analog was first described by Burke and his colleagues (Smyth et al. 1992; Burke et al. 1993) and was subsequently introduced into peptides and evaluated as SH2 ligands in 1994 (Burke et al. 1994; Gilmer et al. 1994). The phosphonate moiety is not only resistant to hydrolysis by adventitious intracellular phosphatases, but the electron-withdrawing effect of the difluoro substituents ensures that the phosphonate is doubly ionized at physiological pH. In addition to the difluoromethylene moiety, a variety of other groups have been used to link the phosphoryl moiety to the aromatic nucleus, including methylene, hydroxymethylene, and fluoromethylene (Burke et al. 1994). The -OPO₃H group on phosphotyrosine has also been replaced with -CH₂COOH (Gilmer et al. 1994; Tong et al. 1998), -OCH₂COOH (Burke et al. 1999), -CHOHCOOH (Beaulieu et al. 1999), -CF₂COOH (Burke et al. 1999), -CH₂SO₃H (Gilmer et al. 1994), -CH₂CH₂COCOCH₃ (Gilmer et al. 1994), and NO₂ (Gilmer et al. 1994). In addition, the phosphoryl group has been directly attached to the aromatic ring (i.e., no bridging group) (Stankovic et al. 1997). A number of geminal analogs [e.g., 21 (Bohacek et al. 2001) and 22 (Kole et al. 1995; Ye et al. 1995)] have been prepared and examined as well. Although the general statement that peptides containing 20 in place of phosphotyrosine possess the greatest affinity for their intended targets may be true, there is sufficient variability among the protein-binding domains that any generalization concerning binding preferences is dangerous. For example, the monocarboxylic acid analogs of phosphotyrosine appear to have reasonable affinity for certain SH2 domains, but are poor inhibitors for the protein tyrosine phosphatase family member PTP1B (Gao et al. 2000).

4.1.4 Proline Analogs

Proline is commonly thought of as a turn-promoting residue. Indeed, a large number of turn-inducing proline mimetics have been described, some of which have been synthetically incorporated into peptides. Sarcosine, also known as N-methyl glycine (CH₃NHCH₂COOH), is a commonly employed proline replacement. Indeed, the latter has been used in substrates for cyclin-dependent protein kinases, enzymes that require a proline residue on its intended substrate to direct phosphorylation to an adjacent serine moiety. For example, Ando and coworkers showed that peptides containing the sequence from vimentin, Leu-Gly-Ser-Ala-Leu-Arg-Arg-Arg-amide, in which the alanine moiety is replaced by either proline or sarcosine, serve as enhanced substrates for the cdc2 protein kinase (V/K 600-fold for Pro-peptide and 90-fold for Sar-peptide versus the Ala-peptide) (Ando et al. 1993). These investigators subsequently examined the specificity of the cdc2 and cdk5 proline-directed protein kinases with respect to a variety of N-substituted proline replacements including, N-methyl alanine, N-methyl leucine, Nmethyl valine, and the four (azetidine carboxylic acid) and six ring (piperidine carboxylic acid) analogs of proline (Ando et al. 1997). Perhaps not too surprisingly, the peptides containing the ring analogs of proline were among the best substrates for these protein kinases, with the proline-containing parent serving as the most efficient substrate. Nevertheless, the sarcosine derivative serves as a substrate as well, albeit somewhat more efficiently for the cdc2 versus cdk5 enzyme. The latter observation is significant since sarcosine is the parent of N-substituted glycine derivatives. Peptides containing the latter ("peptoids") are readily prepared using standard solid-phase peptide synthesis conditions (Figliozzi et al. 1996; Burkoth et al. 2003). Indeed, recent improvements have reduced the coupling time to a few minutes (Olivos et al. 2002). Consequently, it is now possible to prepare large libraries of peptoid-based derivatives in an essentially automated fashion.

Lim and his colleagues have reported that N-substituted amino acids serve as proline replacements in peptide-based ligands targeting both SH3 and WW domains (Nguyen et al. 1998; Nguyen et al. 2000). These investigators demonstrated that the proline selectivity for these domains is due to a preference for N-substituted residues and not simply a function of the rigid cyclic structure that is unique to proline. The SH3 domain from Sem5 recognizes the motif PPPVPPR, whereas the WW domain from Yap prefers GTPPPPYTVG (where the "essential" prolines are underlined). The initial strategy employed a scanning approach in which both essential and nonessential prolines were replaced by either the C^{α} -substituted alanine or the N-substituted sarcosine. Replacement of the essential prolines with sarcosine is well tolerated, whereas introduction of alanine at these sites is not. The three-dimensional structures of these protein recognition domains are known. The ligand binds as a polyproline type II left-handed helix, which contains three residues per turn. The binding grooves on these protein domains can accommodate substituents from the type II helix, but only in a closely packed fashion. The latter can be achieved by a motif in which a C^{α} substituent is adjacent to an N-substituted residue. A small library was prepared that consisted of peptides containing 11 different N-substituted glycines at the two essential proline sites in an SH3-targeted ligand. These were screened against the SH3 domains from Src, Grb2, Crk, and Sem5.



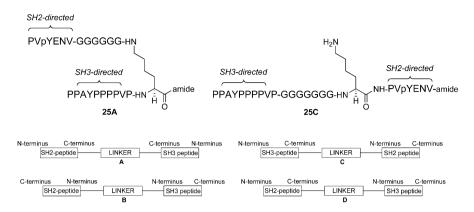
The lead peptide for Grb2, 23, exhibits a K_D of 40 nM, more than 100-fold better than that of the corresponding proline-containing derivative. A subsequently prepared library, containing N-substituted glycine residues with oxygen- and nitrogen-containing substituents, was also evaluated (Nguyen et al. 2000). The latter functionality was chosen for their ability to interact with polar residues that reside near the ligand-binding grooves of the SH3 domain. Compound 24 exhibits a 30 nM \bar{K}_D for the Grb2 SH3 domain and high selectivity versus Crk (300-fold) and Src (1,500-fold). A high-affinity ligand with impressive selectivity was also identified for the Crk SH3 domain. Double proline-substituted derivatives were also prepared, but these ligands failed to show an additive improvement in affinity for their protein targets. The latter is likely because the N-substituted glycines at the two different "essential" proline sites were identified independently of one another. Introduction of an N-derivatized glycine at one position likely induces a conformational change between ligand and protein that influences the interaction at the second proline site. Nevertheless, these results demonstrate that "essential" residues cannot only be replaced with unnatural analogs, but that the latter can furnish dramatically enhanced potency and selectivity.

4.2 Multidomain-Targeting Peptides

Many protein kinases contain two or more independent protein-binding domains. This includes the best-known example, Src, which contains SH1 (active site), SH2, and SH3 domains. Others, such as the LIM-kinase (LIM, PDZ, and active site regions) likewise contain multiple domains (Stanyon and Bernard 1999). The twin issues of potency and specificity can potentially be addressed by simultaneously targeting two protein interaction domains on the same enzyme. First, a single bivalent peptide that concomitantly interacts with two binding domains should display an enhanced affinity for the protein kinase target relative to either monovalent peptide alone. Second, although the sequence homology within closely related domains can be very high, the relative three-dimensional disposition of two protein interaction domains in the intact protein may differ substantially from one protein kinase to the next. Consequently, the linker that connects the two monovalent consensus sequences could play an important role in conveying enzyme selectivity. At this point in time, however, the potential of bivalent (or multivalent) peptides that can interact with two or more protein interaction domains is at an early stage in development and has not yet been fully realized.

One of the first papers to describe the preparation of bivalent ligands for signaling proteins dealt with the SH-PTP2 protein tyrosine phosphatase (Pluskey et al. 1995). The latter contains two SH2 domains. Occupancy of these domains is known to stimulate phosphatase activity. Previous studies had identified peptides SLNpYIDLDLVK and LSTpYASINFQK that specifically target the N-terminal and C-terminal SH2 domains, respectively (Case et al. 1994; Sugimoto et al. 1994). These monovalent species were linked with one another via an aminocaproic acid bridge to create a consolidated derivative, LNpYIDLDLVK-(6-aminocaproic acid)₄-LSTpYASINFQK, that can simultaneously interact with both SH2 domains. The latter does not act as an inhibitor, since it was designed to promote phosphatase activity. Nevertheless, it stimulates the phosphatase by 37-fold, compared with the 9- to 16-fold displayed by the monovalent consensus peptides. In addition, the heterodimeric peptide displays a 60- to 90-fold higher affinity for SH-PTP2 than either monomer peptide alone.

Cowburn, Barany, and their colleagues have described peptides that simultaneously target the SH2 and SH3 domains of Abl (Cowburn et al. 1995). The SH2-targeting sequence (PVpYENV-amide; K_D =2.0±0.2 µM) was attached to the SH3-targeting sequence (PPAYPPPVP; K_D =10.5±0.2 µM) via a lysine side chain as illustrated in **25**. The latter exhibits a K_D of 249±5 nM for the SH2–SH3 domain construct, which is approximately 10- to 80-fold greater than that of individual monovalent consensus peptides. These investigators subsequently prepared a series of bivalent ligands containing the individual monomers in the four possible orientations illustrated in Scheme 1 25*A*–*D* (Xu et al. 1999). The highest affinity ligand, **25***C*, exhibits a K_D of 190 nM for the Abl SH2-SH3 (where LINKER=Gly₇K).

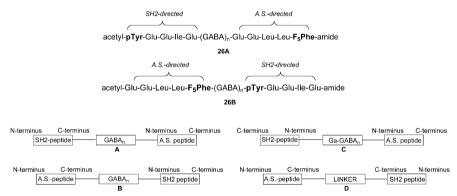


Roques, Garbay, and coworkers prepared peptides that interact with the two SH3 domains of Grb2 (Cussac et al. 1999). As with the bivalent analogs described in this section, the strategy employed coupling two monomer units via a linker. Two SH3-binding monomer units, Val-Pro-Pro-Val-Pro-Pro-Arg-Arg-Arg, were attached to each other via their C-termini using the lysine-based strategy described by Cowburn and Barany. The bivalent species exhibits an affinity (K_D =40 nM) that is two to three orders of magnitude greater than that exhibited by the corresponding monomer (N-terminal SH3 domain: K_D =2.6 µM; C-terminal SH3 domain: K_D =40 µM). These investigators demonstrated that the peptide dimer blocks Grb2-Sos complex formation in cell lysates, can selectively pull down Grb2 from lysate versus other double SH3 domain-containing proteins, and interferes with neurite formation in nerve growth factor-treated PC12 cells.

Miller and his coworkers prepared a series of SH2 domain-assisted *sub*strates for the Abl tyrosine kinase (Pellicena et al. 1998). These investigators found that the presence of an SH2 domain binding sequence appended to the active site-directed sequence enhances substrate efficacy, specifically via a tenfold reduction in K_m . For example, a peptide of the general structure (active site-directed peptide)–(SH2-directed peptide) displays a K_m of $69\pm11 \mu$ M and a V_{max} of $3.0\pm0.1 \mu$ mol/min-mg. The key residue in the SH2directed component is phosphoTyr, which is essential for SH2 recognition. When a Phe replaces this critical residue, the peptide displays a significantly larger K_m of $680\pm90 \mu$ M, yet a V_{max} ($3.0\pm0.1 \mu$ mol/min-mg) that is essentially unaltered. A peptide containing an inversed orientation, namely (SH2-directed peptide)–(active site-directed peptide), likewise displays a relatively low K_m value ($72\pm5 \mu$ M), which is presumably a reflection of enhanced affinity via coordination to the SH2 domain.

Lawrence and his collaborators employed the multidomain targeting approach to create combined active site/SH2 domain-directed inhibitors of Src (Profit et al. 1999; Profit et al. 2001). Peptide-based inhibitors of tyrosine kinases must confront at least two challenges. First, peptides that target the ac-

tive site tend to be exceedingly poor inhibitory agents. Second, although peptides that bind to the SH2 domain are of reasonably high affinity (~low μ M) they have the unintended consequence of activating the kinase. Indeed, by analogy, Shoelson's and Walsh's bivalent ligand, which binds to the two SH2 domains of SH-PTP2, dramatically enhances phosphatase activity. However, an inhibitor that combines SH2 and active site-binding properties should simultaneously display enhanced affinity and inhibitory potency. In addition, bivalent analogs can furnish an assessment of the distance and spatial relationship between the protein interaction domains on the protein under evaluation. Indeed, the Lawrence team referred to their small library (16 compounds) as "molecular rulers." In a manner analogous to Barany and Cowburn, a series of differentially oriented SH2-directed and active site-directed bivalent ligands were prepared. γ -Aminobutyric acid (GABA) residues were employed in the linker region to connect the monovalent ligands. The active site-directed fragment, -Glu-Glu-Leu-Leu-(F5Phe)-, contains pentafluorophenylalanine, which had been previously identified as a nonphosphorylatable tyrosine surrogate (vide supra) (Niu and Lawrence 1997a). The SH2 sequence, -pTyr-Glu-Glu-Ile-, was based on the well-known preference of the SH2 domain from Src for a sequence motif containing pTyr followed by at hydrophobic amino acid at the P+3 position (Songyang et al. 1993).



Of the four possible relative orientations between the SH2- and active site-directed fragments (Scheme 2), only three were prepared (A-C) since the fourth (D) was subsequently ruled out as suboptimal (*vide infra*). The orientation illustrated in C requires that the peptide chains in the two monovalent units run anti-parallel to one another. This reversal of chain polarity was achieved via insertion of a glutaric anhydride (Ga) residue between the SH2-directed sequence and the GABA linker. Orientation B furnished the most effective inhibitors possessing the shortest linkers (optimal: GABA_n= GABA₃). In this particular case, the tyrosine surrogate F₅Phe (occupying the

active site) lies only three residues from the pTyr moiety which resides in the SH2 domain (see 26B).

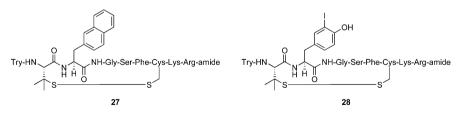
This suggests that the active site region and the SH2 domain are situated close to one another in the active form of the enzyme. The series 26D was not prepared since, even in the absence of a GABA linker, the key F₅Phe and pTyr moieties are positioned at least five residues apart. The latter is suboptimal relative to the relationship in 26B. The lead bivalent analog 26B (where GABA_n=GABA₃) displays an enhanced 120-fold inhibitory potency relative to the simple active site-directed monomer (IC₅₀=13±1 μ M versus IC₅₀= 1590±170 μ M, respectively).

4.3 Structural Modification of Consensus Sequence Peptides

4.3.1 Conformationally Biased Peptides

Cyclization of peptides improves stability against proteases while affording conformational constraints that may enhance inhibitory potency. Research teams at M.D. Anderson and the University of Arizona have explored the efficacy of this structurally distinct class of active site-directed protein kinase inhibitors.

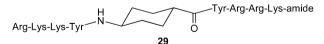
Lam and his colleagues have shown that the Tyr-Ile-Tyr-Gly-Ser-Phe-Lysamide motif serves as an effective Src substrate (Lam et al. 1995) and that the peptide might bind to the active site in the form of a β -turn (Lou et al. 1997). In order to assess the reasonableness of this notion, Lam, Hruby, and their coworkers prepared a series of disulfide bridge-cyclized inhibitory peptides that target Src (Alfaro-Lopez et al. 1998). In addition, these investigators took advantage of the observation that 2-naphthylalanine (Sect. 11) serves as a nonphosphorylatable tyrosine mimetic (Wu et al. 1996).



Compound 27 exhibits an IC_{50} of 1.6 μ M. Interestingly, and somewhat surprisingly, potency was further improved by tenfold when the naphthylalanine was replaced with 3-iodotyrosine (28). This observation stands in marked contrast to the ineffectiveness of 3-iodotyrosine as a tyrosine mimetic in an EGFR-targeted inhibitory peptide (Fry et al. 1994). Peptide 28 is not only an effective Src kinase inhibitor but also exhibits impressive selectivity in favor of Src versus other Src kinase family members (20-fold versus Lck and >1,300-fold versus Lyn). In addition, these investigators found that several of their less potent Src kinase inhibitors (IC₅₀ values 1–3 μ M range) exhibit even better selectivity profiles than **28**.

McMurray, Budde, and coworkers likewise examined the effectiveness of cyclic peptide inhibitors on the Src kinase. These investigators first examined the affinity of a series of cyclic peptide substrates for Src (i.e., the ability of these substrates to block the phosphorylation of poly Glu₄Tyr) (McMurray et al. 1998). One of the lead compounds, cyclo[Asp-Asn-Glu-Tyr-Ala-Phe-Phe⁷-Gln-D-Phe-Pro) displays a K_i of 150 µM, which is nearly identical to its Michaelis constant as a substrate (140 µM). Insertion of an arginine residue at position 7 resulted in a dramatic loss in enzyme affinity, whereas a glutamic acid residue at this site is well tolerated. On the basis of this observation, these investigators concluded that residues at this site are positioned within a positively charged region of the enzyme. Indeed, when Phe⁷ was subsequently replaced with a series of 14 different analogs, the lead inhibitors contained a negatively charged residue at this position [4-carboxyphenylalanine (K_i =0.85 µM) and phosphotyrosine (K_i =1.1 µM)] (Wang et al. 2000). The carboxylphenylalanine-containing cyclic peptide displays an impressive selectivity profile in favor of Src (>100-fold against Yes; >300fold versus Lck; >1,000-fold versus PKA; 1,200-fold versus FGF receptor; 1,800-fold versus Abl; >2,000-fold versus CSK).

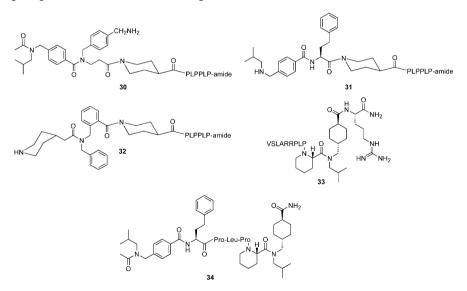
Watterson and his group described a different strategy for topologically biasing an active site-directed peptide (Lukas et al. 1999). Myosin light chain kinase (MLCK) was the target in this particular case. These investigators first identified a nonapeptide sequence, Arg-Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys-amide that exhibits both a remarkable affinity ($IC_{50}=50$ nM) and selectivity (~4,000-fold versus CaM kinase II) for MLCK. Based on the screening results of closely related peptides, in combination with molecular modeling, it was proposed that the peptide might bind to the active site region in an extended conformation. The structural constraints inherent within 4-aminocyclohexanecarboxylic acid were used to promote this desired conformation by inserting the residue at specific sites within the peptide sequence.



The most potent of these derivatives (29) exhibits an IC_{50} (40 nM) similar to that of the parent nonapeptide. Although improved potency and selectivity were not observed versus the already formidable peptide parent, the results in this study suggest that conformational constraints could serve as scaffolds upon which an array of functionality can be appended.

4.3.2 Terminally Modified Peptides

The fatty acid-modified peptides described in Sect. 4.1.2 are examples of terminally modified consensus sequences. Addition of the long alkyl chain furnishes enhanced active site affinity via coordination to ancillary binding pockets. However, it should be possible to access potential sites of interaction that are extensions of the protein-binding pocket with non-natural substituents. Schreiber's group explored this concept utilizing N-terminally modified peptides that target the SH3 domain of Src (Combs et al. 1996). The three-dimensional structure of the protein was employed as a guide to focus structural diversity into a potential binding region adjacent to the site at which a peptide ligand of the SH3 domain is known to reside. Structural diversity was created using a split-and-pool approach. Pro-Leu-Pro-Pro-Leu-Pro-resin was split into 33 equal amounts and each fraction subsequently modified at the peptide N-terminus with one of 32 different non-natural amino acids (plus no residue at all). The fractions were recombined and the process repeated two additional times. The one-bead/one-compound library was then exposed to a phosphatase-modified SH3 domain. Beads possessing high-affinity ligands were visually identified under the microscope using the phosphatase as a colorimetric reporter.



The lead peptide identified in this study, **30**, exhibits a K_D of 3.4 μ M for the Src SH3 domain and is approximately 50-fold more selective for Src versus the corresponding SH3 domain from PI3 kinase. By comparison, the parent peptide, acetyl-Pro-Leu-Pro-Pro-Leu-Pro, exhibits a K_D of greater than 1 mM for Src's SH3 domain. A subsequent study, using a slightly larger set

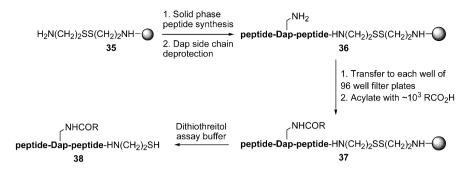
of non-natural monomers and greater structural diversity at each position, identified additional ligands for the Src SH3 domain (31; K_D =0.9 µM) as well as leads for the closely related SH3 domain of Hck (32; K_D =1.0 µM) (Kapoor et al. 1998). Some of these ligands proved to be moderately selective (e.g., 32 is 46-fold more selective for Hck than Src).

The Schreiber group extended the concept of structural diversity to the C-terminus of the SH3-directed ligand as well (Morken et al. 1998). In this instance, the library took the form of Val-Ser-Leu-Ala-Arg-Arg-Pro-Leu-Pro-M3-M2-M1-resin, where M3, M2, and M1 represent 50 different residues, which included 49 monomers plus an omitted residue. The goal of this work was to identify a replacement for the C-terminal Leu-Pro dyad in the parent peptide -Pro-Leu-Pro-Pro-Leu-Pro-. The lead 33 displays a K_D of 2.6 μ M for the SH3 domain from Src. A portion of the C-terminal non-natural component from 31 was then appended, along with the N-terminal non-natural component in 31, to the Pro-Leu-Pro core (34). The latter compound displays a K_D of 1.1 μ M for Src's SH3 domain.

4.3.3 Globally Modified Peptides

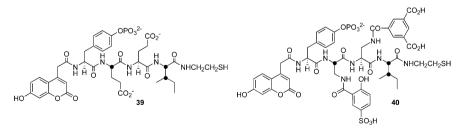
Protein interaction domains have evolved to accommodate specific sequences of preferred amino residues on their protein-binding partner. However, each amino acid site on the bound sequence is limited to a genetically encoded molecular diversity of 20. In reality, the latter is slightly larger due to a small array of possible post-translational modifications. Nevertheless, it is not difficult to imagine that there exist a wealth of potential binding interactions that lie just outside of the reach of this limited set of naturally occurring residues. One strategy to enhance molecular diversity is to prepare a wide assortment of Fmoc and side chain-protected, unnatural, amino acid derivatives and then synthesize the corresponding library of peptides. However, a reasonable 50-fold enhancement in molecular diversity vis-à-vis genetically encoded residues would require the preparation of 1,000 different monomers, a nontrivial task.

Lawrence and his colleagues outlined an approach that creates high diversity at any desired site along a peptide chain using readily available carboxylic acid-containing compounds (Lee and Lawrence 1999). The strategy, as outlined in Scheme 3, employs a consensus sequence peptide containing 2,3-diaminopropionic acid ("Dap"), appropriately inserted at key sites along the peptide chain.



The peptide is synthesized on a disulfide-substituted Tentagel resin. Once prepared, the peptide-resin is distributed in equal amounts to the individual wells of a 96-multiwell plate designed for organic synthesis (i.e., the bottom of each well contains a frit that allows multiple washings without loss of the peptide-resin). Each well is then exposed to one of approximately 1,000 different carboxylic acid compounds. In short, the library is prepared in parallel, thereby obviating the necessity of molecular deconvolution. Once the modification in the Dap residue is complete, any side chain protecting groups on the peptide are removed with trifluoroacetic acid. After multiple washings to remove residual acid, the individual peptides are cleaved from the resin with assay buffer, which contains dithiothreitol. The peptides can then be directly assessed for potency.

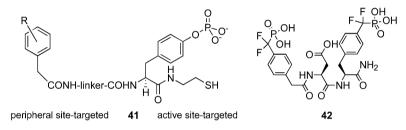
Lawrence and his team employed a structure-based strategy, in combination with the synthetic approach outlined in Scheme 3, to identify high-affinity ligands for the SH2 domain from Lck (Lee and Lawrence 1999, 2000; Yeh et al. 2001). The three-dimensional structure of the Lck SH2 ligand, acetyl-pTyr-Glu-Glu-Ile-amide, bound to its protein target, had been previously reported (Tong et al. 1996). Three sites on the ligand, the N-terminal acetyl moiety and the two Glu side chains, are oriented into regions of the SH2 surface that could potentially accommodate modified analogs of the acetyl and glutamic acid moieties.



The initial library and its subsequent screen furnished compound **39**, which contains a coumarin moiety in place of the former N-acetyl group. The coumarin-derivatized peptide exhibits a K_D of 35 nM for the Lck SH2 domain, approximately two orders of magnitude greater than the parent

peptide aceyl-pTyr-Glu-Glu-Ile-amide. Subsequent identification of the glutamic acid replacements furnished **40**, which displays a K_D of 200 pM, approximately four orders of magnitude better than the starting consensus peptide. An analogous approach was recently used to construct an inhibitor for the α isoform of PKC (Lee et al. 2004). The inhibitor displays a K_i of 800 pM and a selectivity of greater than 400-fold versus other conventional, novel, and atypical PKC isoforms.

Lawrence, Zhang, and their colleagues reported a variation on the Scheme 3 strategy that provided a high-affinity inhibitor for PTP1B (Shen et al. 2001). Once again, a structure-based approach was employed that directed molecular diversity toward potential binding sites on the target protein surface. In this particular case, PTP1B had been previously shown to bind phosphotyrosine at two distinct sites, one at the active site and the other at a position adjacent to the active site (Puius et al. 1997).



A library of the general form **41** was prepared using the disulfide Tentagel resin **35**. Molecular diversity was inserted at the N-terminal and linker positions. The lead compound **42** exhibits a K_i of 2.6 nM for PTP1B and a selectivity of between 1,000- and 10,000-fold versus a panel of fifteen other protein phosphatases.

5 Summary

An exceedingly important, but time-consuming area of drug design is the conversion of consensus recognition sequences into small molecules with drug-like properties. The acquisition of peptidomimetics requires a combination of detailed structural information of the target protein, an intensive synthetic effort, and gifted insight. The rapid development of human immunodeficiency virus (HIV) protease inhibitors stands as a testimony to the fact that, given enough resources, it is possible to successfully create potent small molecule inhibitors that target protein–protein interaction sites (Ab-del-Rahman et al. 2002). However, the sheer number of protein kinases rules out the kind of large-scale assault that transpired in the HIV arena on the protein kinase family as a whole. Nevertheless, at a minimum, a worthwhile

goal is the acquisition of high-affinity reagents for as many signaling proteins as possible. Although consensus sequences represent an obvious starting point, their transmogrification into high-affinity ligands remains an ongoing struggle. Given the large number of possible targets, simple rules or strategies for the conversion of modest-binding peptides into high-affinity reagents are a much sought after commodity. Amino acid analogs, which serve as high-affinity replacements for their natural counterparts, represent one such approach (Sect. 4.1). Alternatively, the notion of targeting two or more protein-protein interaction domains on a single protein kinase, represents a decidedly different tactic (Sect. 4.2). Finally, structurally modified consensus sequences that are either topologically biased or are able to access sites simply unavailable to standard amino acid residues represents a third strategy (Sect. 4.3). Inherent within all of these approaches is the possibility of general applicability to the family of protein kinases. Unfortunately, the route from peptide to a high-affinity species with equally high selectivity is often anything but straightforward. The disadvantage with protein kinases is their large number. However, this disadvantage is also an advantage in that they are all closely related. Consequently, inhibitor design rules that emerge from the study of a few representative members of this large enzyme family may ultimately prove applicable to the family as a whole.

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Part II Pharmacological Potential and Inhibitors of Individual Classes of Protein Kinases

The Paullones: A Family of Pharmacological Inhibitors of Cyclin-Dependent Kinases and Glycogen Synthase Kinase 3

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Abstract Cyclin-dependent kinases (CDKs) regulate multiple pathways such as the cell division cycle, apoptosis, transcription, and neuronal functions. Glycogen synthase kinase 3 (GSK-3) plays a key role in Wnt signaling, cellular response to insulin, cell death, cell proliferation, maintenance of "stemness." Both families of kinases are clearly involved in the onset and development of major human diseases like cancer, neurodegenerative disorders (Alzheimer's and Parkinson's disease, stroke), diabetes, restenosis, viral infections, etc. Homologues of these kinases also regulate the proliferation of unicellular parasites. For these reasons an intensive search for pharmacological inhibitors of these protein kinases has been carried out during the last decade. Numerous small molecular weight compounds have been described that directly compete with ATP for binding to the catalytic site of the kinases. We here illustrate the development of this research area by reviewing the paullones, a family of potent and rather selective inhibitors of CDKs and GSK-3, from their discovery and optimisation to their molecular and cellular characterisation. The potential medical applications of CDK/GSK-3 inhibitors are presented.

Keywords Cyclin-dependent kinase \cdot Glycogen synthase kinase \cdot Cancer \cdot
Neurodegenerative disorders · Paullone · Malaria

Abbreviations	
CDK	Cyclin-dependent kinase
CoMSIA	Comparative molecular similarity indices analysis
GSK-3	Glycogen synthase kinase 3
QSAR	Quantitative structure/activity relationship
MDH	Malate dehydrogenase
SAR	Structure/activity relationship

1 Introduction

1.1 Protein Kinases and Phosphatases

About 30% of human proteins contain covalently bound phosphate. Protein phosphorylation is considered one of the main post-translational mechanisms used by cells to finely tune their metabolic and regulatory pathways. Protein kinases catalyse the phosphorylation of serine, threonine and tyrosine residues of proteins, using ATP or guanosine triphosphate (GTP) as the phosphate donor, while protein phosphatases are responsible for dephosphorylation, the opposite reaction (Fig. 1). With the completion of the sequencing of several important genomes, we are starting to have a better view of the total range of kinases and phosphatases required in an organism. There are about 518+ kinases in man (Adams 2001; Krupa and Srinivasan 2002; Manning et al. 2002; Hanks 2003). Protein kinases are classified according to their primary sequence, the amino acids they target (tyrosine vs

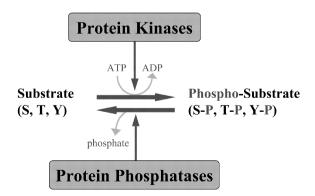


Fig. 1 Phosphorylation and dephosphorylation reactions are catalysed, respectively, by protein kinases and protein phosphatases

serine/threonine) and the nature of their activator. There are approximately 180 phosphatases in the human genome. Protein phosphatases are classified according to the amino acid they dephosphorylate (tyrosine or serine/threonine or dual-specificity phosphatases), and their sensitivity to various inhibitors and ions (PP1, PP2A, PP2B, PP2C) (Jackson and Denu 2001; Kennelly 2001; Sim and Ludowyke 2002; Lyon et al. 2002).

1.2 Protein Phosphorylation and Disease

In view of the importance of phosphorylation in essentially all physiological and cellular events, it is not surprising that abnormal phosphorylation turns out to be a cause or consequence of human disease. A number of diseases actually result from mutations in specific protein kinases and phosphatases (Cohen 2001; Cohen 2002). Of the kinase genes, 244 map to disease loci or cancer amplicons (Manning et al. 2002). In addition, many naturally occurring toxins also exert their effects by altering the phosphorylation state of proteins. Abnormal phosphorylation of proteins is now known to be closely associated with or even a cause of major diseases such as cancers, diabetes, rheumatoid arthritis, Alzheimer's disease, and Parkinson's disease.

These are the reasons why screening for and optimising potent and selective inhibitors of protein kinases and phosphatases has intensified over the last 10 years (review in Adams and Lee 1999; Garcia-Echeverria et al. 2000; Sridhar et al. 2000; Dumas 2001; Bridges 2001; Cohen 2002). However the idea that one could actually target kinases and phosphatases to develop drugs to treat disease was slow to develop. The discovery of the mechanism of action of cyclosporin, the immunosuppressive drug that made transplantation of organs possible, was a strong contributor to this development. Cyclosporin binds to a protein called cyclophilin. The cyclophilin/cyclosporin complex turned out to be a potent and specific inhibitor of PP2B, a calcium/ calmodulin-dependent protein phosphatase. As one of the latest examples, the Novartis compound STI-571, or Gleevec, a potent inhibitor of the Abelson tyrosine kinase, has shown remarkable efficacy in human clinical trials against chronic myelocytic leukaemia (Capdeville et al. 2002a,b). At present, three kinase inhibitors have been approved for clinical use and more than 25 are undergoing clinical trials. The potential of pharmacological inhibitors of kinases and the current status of the inhibitors that are in clinical development have been recently reviewed (Cohen 2002).

1.3 Screening for Protein Kinase Inhibitors

Currently, all major pharmaceutical companies and many biotechnology start-ups run kinase screening assays. Briefly, the screening process involves six different steps (summarised in Doerig et al. 2002): (1) selecting a kinase as an appropriate and disease-relevant target, (2) expressing and purifying the kinase (either a recombinant, tagged enzyme or a native enzyme), (3) configuring the kinase assay using a convenient substrate, (4) assembling a library of compounds (synthetic or natural) or extracts of natural sources (plants, animals, microorganisms), (5) running the screen by assaying the kinase in the presence of the library compounds, (6) characterising the selectivity of the hits by secondary screens on additional targets (and perhaps also on the target cell/organism) and their mechanism of action (enzymology and crystallography approaches). This process is reiterated by synthesis of analogues and testing, leading to the optimisation of the chemical family [structure-activity relationship (SAR) studies], and ultimately allowing the synthesis of a lead compound which can be evaluated on cellular and animal models before clinical trials can be initiated.

In this chapter we would like to present an overview of the strategy developed to identify, optimise and characterise pharmacological inhibitors of two classes of disease-relevant kinases, namely the cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3 (GSK-3). To avoid duplication with numerous reviews published elsewhere, we will here illustrate this field by reviewing a class of well-characterised inhibitors, the paullones.

2 CDKs and GSK-3 as Kinase Screening Targets

We have focused our screening efforts on two families of kinases, the CDKs and GSK-3, as well as PfGSK-3, the GSK-3 homologue kinase in Plasmodium falciparum (the agent responsible for the lethal form of malaria) (Droucheau et al. 2004). CDKs are involved in controlling the cell cycle (CDK1, CDK2, CDK3, CDK4, CDK6, CDK7), apoptosis (CDK1, CDK2, CDK5), neuronal functions and neurodegeneration (CDK5, CDK11), transcription (CDK7, CDK8, CDK9) and exocytosis (review in Morgan 1997; Pavletich 1999; Malumbres et al. 2000; Dhavan and Tsai 2001; Harper and Adams 2001; Maccioni et al. 2001; Malumbres and Barbacid 2001; Knockaert et al. 2002a). GSK-3, an essential element of the Wnt signalling pathway, is involved in multiple physiological processes including cell cycle regulation by controlling the levels of cyclin D1 and β -catenin, dorso-ventral patterning during development, insulin action on glycogen synthesis, axonal outgrowth, HIV-1 Tat-mediated neurotoxicity, apoptosis, Alzheimer's disease characteristic phosphorylation of tau and amyloid- β production, and maintenance of "stemness" (review in Cohen and Frame 2001; Grimes and Jope 2001; Eldar-Finkelman 2002; Kaytor and Orr 2002; Doble and Woodgett 2003).

Potential applications of CDK/GSK-3 inhibitors are being evaluated for the treatment of cancers, neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and stroke, diabetes, restenosis, proliferation of protozoan parasites, and viral infections.

2.1 Kinase Purification and Assay

As a general rule, whenever possible, we are working with native enzymes purified from natural sources rather than with recombinant enzymes. Indeed, recombinant enzyme preparations are sometimes not very active and contain a significant proportion of misfolded, inactive, yet inhibitor-binding, enzyme. For this purpose we have developed several affinity purification methods that allow the easy purification of CDKs and GSK-3. CDK1/cyclin B is purified from starfish oocytes by affinity chromatography on immobilised p9^{CKShs1} (Borgne and Meijer 1996). GSK-3 α/β is purified from pork brain by affinity chromatography on an immobilised axin fragment (Primot et al. 2000) (Fig. 2).

Our kinase assays are based on the radioactive labelling of a substrate with ³³P from [γ -³³P]-ATP (Fig. 2). We are currently running our assays in a robotic system allowing the automated handling of liquid samples, in a 96-well format (Fig. 3). The use of a robotised set-up allows for the running of high numbers of compounds in duplicates and to cope with the generation of large quantities of data. Our 96-well plates are organised in a fairly standard way: 80 samples/plate, the first and last columns being occupied by positive and negative controls (non-inhibited enzyme, background and control in the presence of a potent and previously characterised inhibitor).

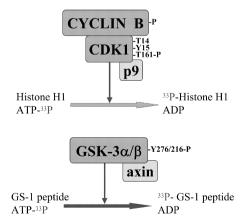


Fig. 2 Principle of the kinase assays. *Top*: active CDK1/cyclin B kinase is purified by affinity chromatography on $p9^{CKShs1}$ -sepharose. It is assayed by measuring the incorporation of radioactive phosphate from radiolabelled ATP into the histone H1 substrate. *Bottom*: active GSK-3 α/β is purified by affinity chromatography on axin-agarose. Its activity is measured using the GS-1 peptide as a substrate

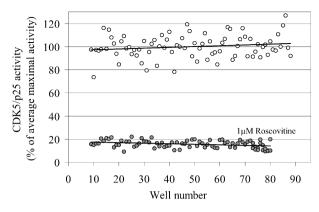


Fig. 3 Validation of the CDK5/p25 assay in a 96-well plate format. All wells, except those from the first and last columns, are filled with kinase assay mix. The kinase assays are run in the absence or presence of 1 μ M roscovitine. The assay allows the unambiguous detection of an inhibitor

As a general rule we first screen the compounds in duplicates at a single concentration (10 μ M for identified compounds; 10 μ g/ml for extracts). Compounds are considered inactive when less than 50% inhibition is observed at this concentration. When a compound displays over 50% inhibition, a second run of assays is carried out to determine the IC₅₀ value from dose–response curves.

2.2 Pharmacological Inhibitors of CDKs and GSK-3

Using these simple methods, a large number of pharmacological inhibitors have been discovered and characterised. Surprisingly many CDK inhibitors, but not all, are also excellent inhibitors of GSK-3 (Leclerc et al. 2001). The properties of these pharmacological inhibitors have been extensively reviewed both for CDKs (Hardcastle et al. 2002; Knockaert et al. 2002a; Sausville 2002; Fischer et al. 2003; Monaco and Vallano 2003) and for GSK-3 (Leclerc et al. 2001; Dorronsoro et al. 2002; Martinez et al. 2002; L. Meijer et al., in preparation). We will thus not duplicate these reviews, but instead summarise the properties shared by these inhibitors in a few lines and provide a more detailed review on the paullones, a family of CDK and GSK-3 dual inhibitors.

Essentially all inhibitors share the following properties:

- They are small molecular weight compounds (<600 Da).
- They are flat, rather hydrophobic, heterocycles.
- They act by competing with ATP for binding to the catalytic site of the kinase.

- They bind in the ATP-binding pocket, located between the small and large lobe of the kinase.
- The interactions with the kinase are predominantly hydrophobic and Van der Waals type.
- The inhibitors also bind through two or three hydrogen bonds with backbone atoms of leucine-83 and glutamic acid 81 (in CDK2) and the corresponding valine-135 and aspartic acid 133 in GSK-3 (Meijer et al. 2003).

3 Paullones as CDK and GSK-3 Inhibitors

3.1 Discovery of Paullones

The National Cancer Institute (NCI) Human Tumor Cell Line Anti-Cancer Drug Screen was established in the late 1980s with the purpose of identifying new anti-cancer drugs (review in Monks et al. 1997). To date the antiproliferative activity of over 70,000 compounds has been measured on the 60 human tumour cell lines panel. Dr. Kenneth Paull conceived a computerised pattern-recognition algorithm, named COMPARE, to allow the identification of new chemotypes that can act through a cellular target similar to that of a reference or "seed" compound (Paull 1992, 1995). In 1997 we used COM-

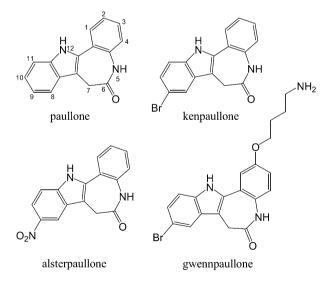


Fig. 4 Chemical structure of the most frequently used paullones. The description of their synthesis has been described in detail: paullone (Kunick 1992), kenpaullone (Kunick 1992; Schultz et al. 1999), alsterpaullone (Schultz et al. 1999), gwennpaullone (Wieking et al. 2002)

PARE with the CDK inhibitor flavopiridol (NSC-649890) as a reference to identify compounds in the NCI collection that might act through CDK inhibition (Zaharevitz 1999). The highest COMPARE correlation coefficient (0.67) was found to be with another CDK inhibitor, olomoucine (NSC-666096) (Vesely et al. 1994). Among five other compounds displaying a correlation coefficient with flavopiridol superior to 0.60, one compound (NSC-664704) was by far the most active against CDK1/cyclin B (IC₅₀: 0.4 μ M). This benzazepinone, 9-bromo-7,12-dihydro-indolo[3,2-*d*]benzazepin-6(5H)-one (Fig. 4), had been synthesised earlier by one of us (Kunick 1992). We renamed it kenpaullone to honour the memory of Dr. Kenneth Paull. Paullone was the name given to the unsubstituted ring structure.

3.2 Molecular Mechanism of Interaction of Paullones with Kinases— Structure/Activity Relationship Studies

Kinetic studies revealed that paullones inhibit kinases by competition with ATP for binding to the catalytic site (Zaharevitz et al. 1999). Since the initial

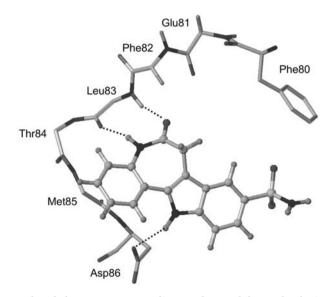


Fig. 5 Hydrogen bonds between CDK1/cyclin B and a 9-sulphamoyl-substituted paullone docked to the ATP-binding pocket. This illustration was created using SYBYL (Version 6.7, Tripos Inc. St. Louis, Missouri 63144, USA), employing the CDK1/cyclin B homology model developed by R. Gussio (Gussio et al. 2000). The manual docking and the subsequent minimisation process, performed using the MAB force field implemented in the program MOLOC (Gerber 1998), were carried out by T. Lemcke, University of Hamburg. To improve clarity of the illustration, the side chains of the amino acids glutamic acid-81 through methionine-85 are not depicted. The *dotted lines* represent the hydrogen bonds formed between the paullone molecule and the amino acids leucine-83 and aspartic acid-86. (This figure comes from the dissertation thesis of T. Pies)

discovery of paullones, more than 60 analogues have been synthesised, leading to compounds active in the nanomolar range (Schultz et al. 1999; Kunick et al. 2000; Leost et al. 2000). In addition, this SAR study has provided some information on the mechanism of interaction of paullones with their kinase targets. Recently, 3D-QSAR CoMSIA models were developed for the inhibition of CDK1/cyclin B, CDK5/p25 and GSK-3 β by the paullones family (Kunick et al. 2004). The models were based on the kinase inhibition data of 52 paullones, docked into the ATP-binding site of a CDK1 homology model (Gussio et al. 2000) (Fig. 5). Recently, alsterpaullone has also been crystallised with GSK-3 β (Bertrand et al. 2003). The co-crystal structure confirms that alsterpaullone is binding in the ATP-binding pocket. The interactions include hydrogen bonds with aspartic acid 133 and valine 135, as observed for other inhibitors recently crystallised with GSK-3 β , 6-bromoindirubin-3'-oxime (Meijer et al. 2003), indirubin-3'-oxime, I-5 and staurosporine (Bertrand et al. 2003) and the thiazole AR-A014418 (Bhat et al. 2003) (see review in Fischer 2003).

3.3 Selectivity of Paullones

Running the initial hits on a small panel of 25 enzymes provided a first view on the selectivity of paullones as shown in Table 1. Clearly paullones appear to be rather specific inhibitors (when compared with staurosporine or flavopiridol, for example) which display exquisite selectivity for CDKs (Schultz et al. 1999; Zaharevitz et al. 1999) and GSK-3 (Leost et al. 2000). Using a set of 28 kinases, Bain et al. (2003) confirmed the CDK/GSK-3 selectivity of paullones and showed that the src-family tyrosine kinase LCK was somewhat sensitive to paullones, and, surprisingly, that kenpaullone was more selective than alsterpaullone. It was suggested that the use of roscovitine (Meijer et al. 1997) and kenpaullone may be useful for identifying substrates and functions of CDKs, while the combined use of kenpaullone and lithium may be useful for identifying substrates and functions of GSK-3 (Bain et al. 2003).

However, this time- and money-consuming kinase panel approach has also strong limitations, since most existing kinases (518+) have not been tested. Other potential, non-kinase, targets are not even considered. This is why we recently developed an affinity chromatography method using immobilised compound to "fish out" the real targets of a defined compound (Fig. 6) (Knockaert et al. 2002b; Knockaert and Meijer 2002). This method has also been illustrated recently with the p38 kinase inhibitor SB 203580 (Godl et al. 2003).

Gwennpaullone was thus covalently bound to agarose beads, and extracts of various cell types and tissues were run on this matrix. Bound proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting and microsequencing of inter-

Protein kinases	Ken paullone	Alster paullone
	Inhibition potency	Inhibition potency
GSK-3β	0.023	0.004
CDK1/cyclin B	0.40	0.035
CDK2/cyclin A	0.68	0.015
CDK4/cyclin D1	>100	>10.0
CDK5/p25	0.85	0.040
erk1	20.0	22.0
erk2	9.0	4.5
c-raf	38.0	10-100
MAPKK	>100	>100
c-Jun N-terminal kinase	>100	10-100
Protein kinase C α	>100	>100
Protein kinase C β 1	>100	>100
Protein kinase C β 2	>100	>100
Protein kinase C γ	>100	>100
Protein kinase C δ	>100	>100
Protein kinase C ϵ	>100	>100
Protein kinase C η	>100	>100
Protein kinase C ζ	>100	>100
cAMP-dependent PK	>100	7
cGMP-dependent PK	>100	>100
Casein kinase 1	>100	>100
Casein kinase 2	20.0	>100
Insulin receptor Tyr kinase	>100	>100
c-src	15.0	n.t.
v-abl	>100	n.t.

Table 1 Investigation of the selectivity of paullones by the classical "enzyme panel" method. Kenpaullone and alsterpaullone are tested for their effects on a series of purified kinases and their IC_{50} values (expressed in μ M) are determined from the dose-response curves. A ranking, in accordance with IC_{50} values, highlights the inhibition potency of the paullones on various kinases

n.t., Not tested.

nal peptides. This analysis confirmed that GSK-3 α and GSK-3 β are prominent targets of paullones. Quite unexpectedly, mitochondrial malate dehydrogenase (MDH) was found to bind to the paullone matrix. This was observed with various biological sources ranging from sea urchin eggs, *Xenopus* oocytes, mammalian brain and various cell lines, to unicellular parasites (*Leishmania*).

MDH catalyses the conversion of malate into oxaloacetate. It plays a role in a variety of essential metabolic pathways such as the citric acid (Krebs) cycle, the tricarboxylic acid cycle, gluconeogenesis and amino acid synthesis. Paullones inhibit purified MDH by competing with NAD (Knockaert et al. 2002b). The interaction of paullones with MDH may contribute to the anti-mitotic properties of these compounds, although this needs to be investigated. In this respect, one of the targets of E7070, a sulphonamide com-

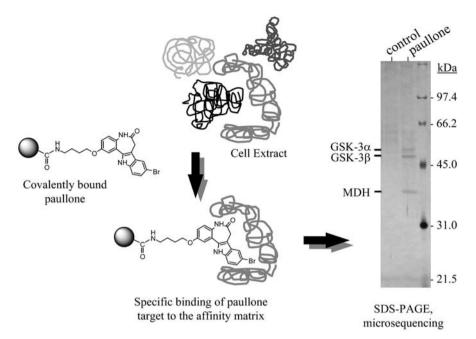


Fig. 6 Affinity chromatography purification of paullone targets. Gwennpaullone is immobilised to agarose beads. Cell extracts are incubated with this matrix as well as a control matrix. After extensive washing, the matrix-bound proteins are resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and silver-stained. The proteins are then excised from the gel and identified by microsequencing of internal peptides. (Aadapted from Knockaert and Meijer 2002)

pound currently undergoing phase II clinical trials against cancer, was recently identified as cytosolic MDH (Oda et al. 2003). E7070 is suggested to act by competing with NADH binding. Cytosolic and mitochondrial MDH may thus constitute new targets for drugs with anti-tumour properties.

3.4 Cellular Effects of Paullones

Paullones have been tested as potential anti-tumour agents. They indeed inhibit mammalian cell proliferation in culture (Schultz et al. 1999; Zaharevitz et al. 1999; Gussio et al. 2000) with an accumulation of cells both in G1 and G2, as would be expected from an inhibition of both CDK1 and CDK2. However, the fact that paullones inhibit CDKs in vivo still remains to be demonstrated. Paullones also inhibit the proliferation of *Leishmania mexicana* (Knockaert et al. 2002). Two potential targets were purified from this unicellular parasite using gwennpaullone agarose beads, and one of them was identified as mitochondrial MDH (Knockaert et al. 2002), the other as a new MAP kinase-like protein (J. Mottram, personal communication).

A recent study using the leukaemia Jurkat cell line showed that alsterpaullone perturbs mitochondrial membrane potential, induces activation of several caspases (caspase-9, then caspase-8 and caspase-3) and triggers apoptosis (Lahusen et al. 2003). The molecular mechanisms beyond this apoptosis effect are still unclear. Whether inhibition of mitochondrial MDH plays a role in the mitochondrial effects of alsterpaullone, and the subsequent induction of apoptosis, remains to be determined.

Paullones are rather potent inhibitors of the nervous system kinase CDK5 in vitro. To evaluate whether this was true in vivo, we investigated the effects of alsterpaullone on the phosphorylation of DARPP-32 on threonine 75, a CDK5-selective site (Bibb et al. 1999), using isolated brain striatum slices. Alsterpaullone inhibited DARPP-32 threonine 75 phosphorylation in a dose-dependent manner, indicating its ability to target CDK5 in a cellular setting (Leost et al. 2000).

As described above, GSK-3 α and GSK-3 β are also two major targets of paullones in vitro. To demonstrate that they are also in vivo targets, we have analysed the effects of kenpaullone and alsterpaullone on SH-SY5Y cells in culture (Fig. 7). As expected, kenpaullone and alsterpaullone, even more potently, induce an accumulation of β -catenin, a direct consequence of its stabilisation by dephosphorylation. Increased dephospho- β -catenin (detected with an antibody that cross-reacts with β -catenin only when it is not phosphory-

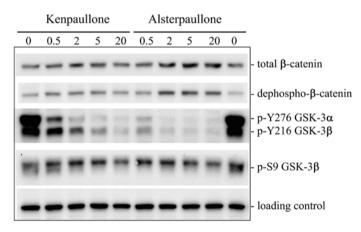


Fig. 7 Paullones are selective GSK-3 inhibitors in cell cultures. SH-SY5Y cells were untreated (0) or exposed to 0.5–20 μ M kenpaullone or alsterpaullone for 24 h. Proteins were then separated by SDS-PAGE followed by Western blotting with antibodies directed (*top to bottom*) against β -catenin, dephospho- β -catenin, phospho-tyrosine 276 (GSK-3 α)/-tyrosine 216 (GSK-3 β), phospho-serine 9 GSK-3 and a loading control (non-specific band detected with the dephospho- β -catenin). (Courtesy of Dr. Xiaozhou P. Ryan)

lated on GSK-3 specific sites) is also seen with both paullones. Finally, both paullones inhibit the phosphorylation of GSK-3 α and GSK-3 β on tyrosine 276 and tyrosine 216, respectively. Phosphorylation of these sites is directly involved in GSK-3 activation, and their inhibition by paullones (through a yet-unknown mechanism) further contributes to GSK-3 inhibition.

Recently, the GSK-3 inhibitory property of kenpaullone has been used to support data demonstrating that GSK-3 α regulates the production of amyloid- β peptides (Phiel et al. 2003). Amyloid- β peptides derive from the amyloid precursor protein through the proteolytic action of β - and γ -secretases. Amyloid- β peptides accumulate and aggregate in Alzheimer's disease, and the formation of amyloid plaques is thought to play a major role in the development of the disease (De Strooper and Woodgett 2003). Very interestingly, both lithium (millimolar concentrations), a classical inhibitor of GSK-3, and kenpaullone (micromolar concentrations), but not roscovitine (inactive on GSK-3), inhibit the production of amyloid- β peptides in cell lines (Phiel et al. 2003). Depletion of GSK-3 α by RNAi together with overexpression studies confirm that GSK-3 α is required for the generation of amyloid- β peptides.

Hyperphosphorylation of the microtubule-binding protein tau, and its subsequent aggregation in paired helical filaments (neurofibrillary tangles) is also a landmark of Alzheimer's disease (De Strooper and Woodgett 2003). GSK-3 and CDK5 are two major kinases implicated in abnormal tau hyperphosphorylation. Using Sf9 cells overexpressing human tau, we found that alsterpaullone is able to inhibit tau phosphorylation on epitopes which represent major GSK-3 phosphorylation sites detected in Alzheimer's disease (cross-reacting with PHF-1 and AT100 antibodies) (Leost 2000).

3.5

Future Development of Paullones

Although numerous analogues of paullones have been synthesised, there is probably space for improvement in both efficacy and selectivity. This would be greatly guided by the co-crystallisation of paullones with a CDK or GSK-3. Hopefully paullones can be designed that are mono-specific for CDKs, GSK-3 or MDH inhibition. This will then allow us to relate the observed cellular effects of paullones to inhibition of a specific molecular target.

Paullones also need to be improved with respect to their solubility. Indeed, like most kinase inhibitors, paullones bind by hydrophobic interactions within the ATP-binding pocket of kinases. They are thus quite hydrophobic and therefore poorly soluble in aqueous media. As this problem could seriously preclude their clinical development, ways to improve the solubility of paullones must be identified. There are several possible solutions, such as the development of water-soluble analogues, nano-encapsulation, insertion into liposomes, and the formation of water-soluble pro-drugs.

4 Conclusion

The identification of paullones as a family of CDK inhibitors has been followed by several unexpected findings, specifically their stronger affinity for GSK-3, and their interactions with mitochondrial MDH. This, in fact, has opened the way to alternative applications beyond the initial anti-cancer application (Zaharevitz et al. 1999), which had not been initially predicted, namely the use against neurodegenerative disorders. As CDK/GSK-3 dual specificity inhibitors, paullones could be highly advantageous, since both CDK5/p25 (Cruz et al. 2003; Noble et al. 2003) and GSK-3 (Caricasole 2003; De Strooper and Woodgett 2003; Phiel et al. 2003) have been shown to play essential roles in the development of Alzheimer's disease. CDK5 has also been shown to mediate dopaminergic neuron loss in Parkinson's disease (Smith et al. 2003). Recently modulation of N-methyl-d-aspartate receptors by CDK5 has been shown to represent a primary event underlying the ischaemic injury of CA1 pyramidal neurons as observed in stroke (Wang et al. 2003). Therefore CDK inhibitors like paullones may have an input in the treatment of Parkinson's disease and stroke as well. Finally, as GSK-3-selective drugs, paullones may find applications in the treatment of diabetes type 2 and as a tool to maintain undifferentiated embryonic stem cells (Sato et al. 2004).

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Pharmacological Potential of p38 MAPK Inhibitors

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Abstract A key component of the intracellular signaling pathways involved in cellular response to environmental stress and inflammatory cytokines is the p38 family of mitogenactivated protein kinases (MAPKs). Of the four isoforms of the p38 family of MAPKs identified thus far, $p38\alpha$ is the most characterized enzyme. Since the discovery of $p38\alpha$ MAPK as a target of a series of compounds that inhibited the production of inflammatory cytokines, an intense effort has been applied to further identify, develop, and refine highly potent and selective inhibitors of this enzyme. In addition, availability of p38a MAPK inhibitors has allowed the investigators to dissect this signaling pathway and to examine its role in various pathologies. A large body of biochemical as well as genetic evidence indicates a critical role of p38 α MAPK in both the production of inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) and subsequent signaling initiated in response to these cytokines. This suggests that inhibition of $p38\alpha$ MAPK pathway will have utility in pathological settings where tissue inflammation and pro-inflammatory cytokines have been implicated. Indeed, several $p38\alpha$ MAPK inhibitors have been shown to be efficacious in preclinical animal models of a variety of diseases, including rheumatoid arthritis, pulmonary diseases, neuronal protection, and cancer. In the past few years, several groups have advanced inhibitors into early clinical studies for rheumatoid arthritis, but none thus far has reached the critical phase III efficacy stage. In this chapter, we review the p38 MAPK pathway and pharmacological potential of p38 α MAPK inhibitors in various pathologies with particular emphasis on inflammatory diseases.

Keywords p38 MAPK · Inhibitors · Inflammation · Interleukin 1 · Tumor necrosis factor · Cytokines · Rheumatoid arthritis · Respiratory · Neuronal

1 Introduction

The ability of living cells to respond to the multitude of signals emanating from its environment rests with a variety of signaling pathways inside the cell. The components involved and their assembly in a pathway are dependent upon the type, duration, and magnitude of the signal and ensure the appropriate integrating and processing of the signal resulting in a stimulusspecific response. One of the major intracellular signaling pathways is the mitogen-activated protein kinase (MAPK) pathway. A central component of this pathway is the MAPKs. The MAPK signaling cascade consists of three protein kinases (Pearson et al. 2001), MAPK and two upstream components, MAPK kinase (MAPKK or MKK) and MAPKK kinase (MAPKKK) (Fig. 1). Three MAPK pathways have so far been described in mammalian cells. The first to be discovered was the extracellular signal-related kinases, ERK1 and ERK2. Subsequently, c-jun amino terminal kinase (JNK) and p38 MAPK

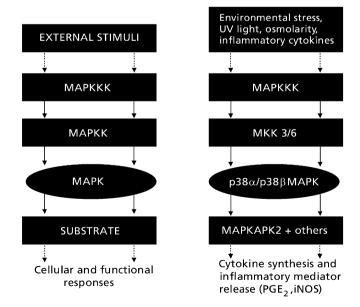


Fig. 1 p38 MAPK pathways with activators and substrates

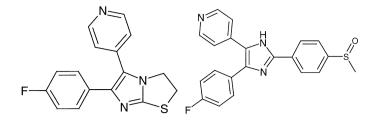
were described. These kinases exhibit between 60% and 70% amino acid identity to one another. Differences have been demonstrated in the sequence and size of their activation loop as well as their responsiveness to different stimuli. Each of the MAPK sub-families has been shown to consist of multiple isoforms and sub-family members. Activation of the kinases occurs through the dual phosphorylation of Thr and Tyr residues in a "TXY" motif (X being Glu, Pro or Gly in ERKs, JNKs and p38 respectively) by a dual specificity MAPKK (Pearson et al. 2001). A serine threonine kinase, MAP-KKK, is responsible for the phosphorylation of MAPKK. As previously stated, the MAPK can be differentiated by their responsiveness to stimuli. In general, the ERKs have been shown to respond to mitogenic and proliferative stimuli, whereas the JNKs and p38 MAPKs respond to environmental stresses such as UV light, heat, osmotic shock and exposure to inflammatory cytokines (Pearson et al. 2001).

The elucidation of the p38 MAPK pathway began almost a decade ago, when the murine p38 was identified as a major phosphoprotein activated as a result of bacterial lipopolysaccharide (LPS) challenge (Han et al. 1994). Shortly following this discovery, human p38 was identified as the molecular target for members of the pyridinylimidazole class of compounds which were known to inhibit the biosynthesis of inflammatory cytokines in LPS-stimulated human monocytes (Lee et al. 1994). The extensive use of bioinformatics has led to the identification $p38\beta2$, $p38\gamma$, and $p38\delta$. Of the four isoforms of human p38 so far described, p38 α is both the best characterized and potentially the most relevant in its involvement in the inflammatory response. Tissue distribution data for p38 α and β 2 demonstrate them to be widely expressed across a variety of tissues. Functionally, however, they appear to be distinct. For example, while both $p38\alpha$ and $p38\beta2$ were shown to be elevated in a mouse model of ventricular hypertrophy, increased $p38\alpha$ activity was associated with cardiomyocyte apoptosis, whereas elevated $p38\beta2$ led to an induction of cardiomyocyte hypertrophy (Braz et al. 2003). Much less is known about the functional role of the other two kinases, p38 γ and δ . A wide tissue distribution in both adult and developing tissues has been shown for $p38\delta$, whereas p38y showed a more restricted distribution in skeletal muscle. A possible role for p38y in cardiac pathophysiology has recently been postulated with the discovery of its expression in normal and diseased human heart tissue and in normal and hypertrophic rat myocytes (Court et al. 2002).

Activation of p38 α and β 2 MAPK occurs through the dual phosphorylation of Thr180 and Tyr182 by the upstream MAPKK, MKK6. Another MAPKK, MKK3 has also been shown to activate p38 α MAPK. MKK3/6 are, in turn, activated by several MAPKKK in response to a variety of stimuli (Adams et al. 2001a; Kyriakis and Avruch 2001) (Fig. 1). These multiple activation pathways serve to illustrate the complexity of this cascade. Recently, a MAPKK-independent pathway of p38 α MAPK activation has been described. This involves the transforming growth factor- β -activated protein kinase-1 (TAK-1) binding protein 1, TAB1 (Ge et al. 2002). Using a yeast two-hybrid system, TAB1 was shown to associate with $p38\alpha$ and induce its intramolecular autophosphorylation. The substrates of the p38 MAPK include other kinases, transcription factors, and cytosolic proteins. Various protein phosphatases including protein phosphatase 2A have been shown to dephosphorylate p38 MAPK, resulting in its downregulation. p38 α MAPK is also downregulated through the dephosphorylation activity of MAPK phosphatase (MKP)-7 and MKP-5 (Theodosiou et al. 1999; Masuda et al. 2001; Tanoue et al. 2001).

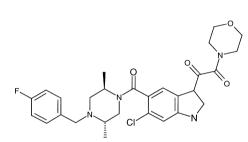
2 Discovery of p38 Inhibitors

Lee et al. first reported a series of bicyclic imidazoles that inhibited both 5-lipoxygenase/cyclooxygenase (LO/COX) and inflammatory cytokines production (Lee et al. 1988, 1993, 1994, 1999). SKF 86002 was one of the first compounds that exhibited potent anti-inflammatory activity and suppression of inflammatory cytokines. In 1995, Gallagher et al. described SB 203580 and other 2,4,5-triaryl imidazoles that were used as pharmacological tools to identify p38 MAPK as the molecular target of these compounds. SB 203580 has since been widely used as a pharmacological tool to elucidate and understand p38 MAPK pathways and p38 MAPK's physiological role (Lee et al. 1994; Gallagher et al. 1995, 1997). These early compounds, however, were not very selective, and further modification of the imidazole template led to improved selectivity and developability characteristics. For example, imidazoles with substitution of two-substituted pyridine in place of pyridine resulted in reduced cytochrome P450 inhibition (Adams et al. 1998). Further improvement of kinase selectivity and potency was accomplished by several groups (Boehm et al. 1996; Liverton et al. 1999; Adams et al. 2001b). The central imidazole core has been replaced by other heteroaryl (Jackson and Bullington 2002) as well as novel non-aryl-pyridinyl scaffolding (Cirillo et al. 2002). The key interaction with a majority of these inhibitors and p38 MAPK is between a carbonyl group acting as a hydrogen bond acceptor to Met 109 in p38 α MAPK and a fluorinated phenyl group fitting in the aryl selectivity pocket of the compound. As is the case with imidazoles, these diverse sets of new compounds also potently inhibit $p38\alpha$ in an ATP-competitive manner. However, recently scientists at Boehringer Ingelheim have identified N-N'-diaryl ureas exemplified by BIRB-796 that binds to the ATP binding site and kinase specificity pocket but not in an ATP-competitive manner, forcing a conformational change in p38 α MAPK. However, unlike imidazoles, BIRB-796 binds in a time-dependent manner and has slow association kinetics of binding (Pargellis et al. 2002; Regan et al. 2002). Some recent examples of indoles (Scios, WO0071535), tetrasubstituted imidazoles (Merck, WO9712876), and heteroaryl fused pyrimidinones (GlaxoSmithKline, WO02059083 and Merck, WO02058695) from patent literature are given in Fig. 2. A detailed descrip-

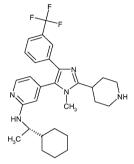


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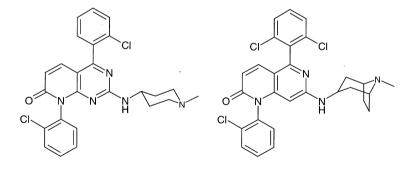
SB-203580



1 Scios (WO0071535)

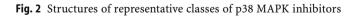


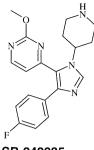
2 Merck (WO9712876)



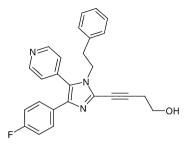
3 GSK (WO02059083)

4 Merck (WO02058695)

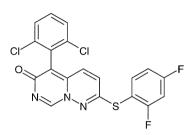


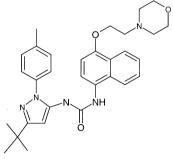


SB-242235



RWJ-67657





VX-745

BIRB-796

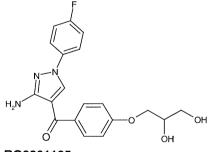




Fig. 2 (continued)

tion of the discovery of p38 inhibitors and their structure activity relationships can be found in Kumar et al. (2003).

Several p38 MAPK inhibitors have reached early clinical studies. SB 242235 was tested in healthy human volunteers. A dose-dependent inhibition of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-8 production was observed in an ex vivo study with isolated PBMNCs from drug treated individuals (Fullerton et al. 2000). Similarly, the effect of RWJ65657 was evaluated on clinical symptoms and cytokine production in response to LPS in healthy human volunteers. Both flu-like clinical symptoms and an increase in serum levels of TNF α , IL-6, and IL-8 were dose-dependently inhibited (Fijen et al. 2001). VX745 from vertex has been evaluated in patients with active rheumatoid arthritis. Although a significant effect of drug was observed on the clinical arthritis score, it was associated with elevated liver enzymes (Weisman et al. 2002). Other compounds reported to be in clinical studies are BIRB796, RO-320-1195 and Scio-469 (Polmar and al. 2002). The structures of several p38 MAPK inhibitors undergoing clinical studies are presented in Fig. 2.

3 Regulation of Cytokine Expression

Evidence for the pivotal role of the $p38\alpha/MAPK$ -activated protein kinase 2 (MAPKAP K2) pathway in cytokine production and signaling has been obtained from mouse genetic studies. Embryonic stem cells from p38 α knockout mice demonstrated both a reduced capacity to produce IL-6 in response to IL-1 and a reduction in MAPKAP K2 activation in response to chemical stress (Allen et al. 2000). Additionally, MAPKAP K2-null mice exhibited a diminished ability to produce both IL-1 and IL-6 (Kotlyarov et al. 1999). The mechanisms involved in this inhibition appear to involve activity at both the levels of transcription and translation. The 3' UTR (untranslated) regions of the mRNA for inducible cytokines such as IL-1, IL-8, and TNF contain an AU-rich element that has been shown to be responsible for their short half-life (Caput et al. 1986; Shaw and Kamen 1986). Under normal physiological conditions it is believed that these AU-rich regions are occupied by AU-binding proteins, rendering them non-translatable. It is hypothesized that these AU-binding proteins become phosphorylated in a p38 α MAPK-dependent manner in response to appropriate inflammatory stimuli, such as LPS, resulting in the stabilization and translation of these mRNAs. Since MAPKAP K2 deficiency is also able to block the production of these short-lived mRNAs, it appears that $p38\alpha$ MAPK is indirectly responsible for stability of these short-lived cytokine mRNAs (Winzen et al. 1999; Ming et al. 2001). Therefore, the current working hypothesis is that the $p38\alpha/$

Compound	Company	Study/model	Reference(s)
SB-203580	GlaxoSmithKline	AA rat and CIA mice, ischemia/reperfusion	Badger et al. 1996; Ma et al. 1999;
		in rat heart, follicular lymphoma,	Elenitoba-Johnson et al. 2003;
		rat spinal injury	Horiuchi et al. 2003
SB-242235	GlaxoSmithKline	AA rats	Badger et al. 2000
Selective imidazole (L-167307)	Merck	AA rats	Liverton et al. 1999
RWJ 67657	RW Johnson Pharmaceutical	AA rats	Wadsworth et al. 1999
Pyridinyloxazole inhibitor	Novartis	CIA in rats	Revesz et al. 2000
RPR-200765A	Aventis	Streptococcal cell wall-induced arthritis in rats	McLay et al. 2001
RPR-238677	Aventis	Streptococcal cell wall-induced arthritis in rats	McKenna et al. 2002
BIRB 796	Boehringer Ingelheim	LPS challenge in mice	Regan et al. 2002
	Pharmaceuticals		
FR167653	Fujisawa Pharmaceuticals	Ischemia/reperfusion of lung and liver in rats	Kawashima et al. 2001; Kobayashi et al. 2002; Nishikawa et al. 2003
SB-239063	GlaxoSmithKline	Cardiac hypertrophy and dysfunction in rats, cerebral focal ischemia in rats, balloon injury in rabbit	Barone et al. 2001; Behr et al. 2001; Ju et al. 2002

 Table 1
 Summary table of pre-clinical pharmacology studies with various p38 MAPK inhibitors

MAPKAP K2 pathway is involved in the phosphorylation of these AU-binding proteins (Neininger et al. 2002; Frevel et al. 2003).

4 Data with p38 Inhibitors

The discovery that members of the pyridinylimidazole class of compounds could inhibit the biosynthesis of inflammatory cytokines in LPS-stimulated human monocytes has led to an abundance of preclinical studies which point to a critical role for $p38\alpha$ MAPK in the inflammatory process. Studies have investigated the potential clinical value of inhibiting this pathway in therapeutic areas including rheumatoid arthritis, pulmonary disease, and neuroprotection. The diversity of these studies is exemplified by the listing in Table 1.

4.1 Rheumatoid Arthritis

In addition to its role in inflammatory cytokine synthesis, the p38 α MAPK pathway is also involved in the induction of other genes encoding inflammatory mediators including COX-2 and inducible nitric oxide synthase (iNOS) (Guan et al. 1998; Badger et al. 2000). These findings have resulted in much of the preclinical and clinical research being focused upon its role in the chronic inflammatory joint disease rheumatoid arthritis (RA). RA is characterized by the infiltration of immunocompetent cells into the synovial tissue and fluid, proliferation of synovial fibroblasts, and the formation of pannus tissue, which invades and degrades the articular cartilage and subchondral bone. The etiology of this disease is unknown. However, many of the cellular and molecular mechanisms underlying the inflammation and associated degradation of the extracellular matrix of the articular cartilage and subchondral bone are becoming more clearly understood (Lee and Weinblatt 2001). A large body of evidence has emerged over the last two decades to implicate the inflammatory cytokines TNF and IL-1 as playing a pivotal role in the pathogenesis of this disease. As illustrated by Fig. 3, these pleiotropic inflammatory cytokines have been shown to promote cartilage degradation and bone resorption in vitro (Arend and Dayer 1995), stimulate the release of prostaglandin E₂ and collagenase from synovial fibroblasts (Dayer et al. 1985), and induce the expression of adhesion molecules on vascular endothelium and inflammatory cells leading to diapedesis (Cavender et al. 1987). Biological agents that effectively neutralize the activity of these cytokines have been shown to be highly effective in the clinic (Lee and Kavanaugh 2003). The chimeric anti-TNF antibody Remicade and the TNF-R-Fc fusion protein Enbrel both bind to TNF and prevent it from binding to its receptor

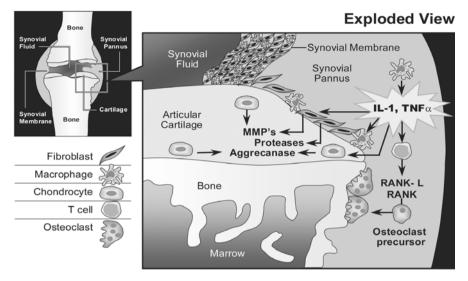


Fig. 3 A simplified diagrammatic representation of the rheumatoid joint (*upper left*). An exploded view of the major cellular and molecular events occurring within the synovial pannus is shown in the *large panel*. The central role of pro-inflammatory cytokines IL-1 and TNF in the expression of downstream mediators of inflammation and joint damage is highlighted. (Reprinted from Kumar et al. 2001a, with permission from Elsevier)

and exerting its biological effects. Anakinra, the IL-1 receptor antagonist (IL-1RA), binds to the IL-1 receptor but does not signal, thereby inhibiting the biological activity of IL-1. However, these therapies are relatively expensive and need to be parenterally administered. It is also clear from the clinical studies that not all patients respond to these therapies (Lee and Kavanaugh 2003). Preclinical data strongly suggest that dual antagonism of both of these mediators would offer a more synergistic benefit than antagonism of either cytokine alone (Bendele et al. 2000). Therefore, an orally active agent that can inhibit either the production or activity of these inflammatory mediators may offer superior therapeutic benefit in the treatment of RA.

4.1.1 In Vitro Data Supporting a Role for p38 α MAPK in RA

Inflammatory cytokine production from many of the cell types present in the rheumatoid joint has been shown to be dependent on the p38 α MAPK pathway. The ability of monocytes, synovial fibroblasts, chondrocytes, and osteoblasts to generate IL-6 and IL-8 was shown to be attenuated by p38 α MAPK inhibition (Suzuki et al. 2000; Adams et al. 2001a). It is of note that p38 α MAPK inhibition does not result in the prevention of proteoglycan loss from IL-1-stimulated articular cartilage (Badger et al. 2000). However, a significant inhibitory effect on iNOS production was demonstrated. Both IL-1 and TNF can stimulate osteoclast-mediated bone resorption through their ability to act directly or indirectly on the osteoclast or their precursors. This indirect activation results from an upregulation of the receptor activator of nuclear factor (NF)- κ B ligand (RANK-L), an important osteoclast differentiation, activation, and survival factor present on osteoblasts and stromal cells. Osteoclast-mediated bone resorption stimulated either by IL-1, TNF, or RANK-L have all been shown to be attenuated by p38 α MAPK inhibition (Matsumoto et al. 2000; Kumar et al. 2001b). These data offer support to the hypothesis that inhibition of p38 α MAPK may not only provide effective anti-inflammatory therapy through their attenuation of cytokine release but may also offer joint protection.

4.1.2 In Vivo Data Supporting a Role for p38 MAPK in RA

The p38 MAPK inhibitor SB 203580 has been used extensively to study the therapeutic potential of this approach in RA preclinical models. Badger and co-workers (1996) demonstrated the anti-inflammatory effects of this molecule in the adjuvant arthritic (AA) rat model of inflammatory joint disease. They reported that it was effective at reducing paw inflammation in the AA rat with an optimum dose of 60 mg/kg administered in a prophylactic dosing regimen. Evidence of a joint-protective effect was also noted using measurements of bone mineral density and histology. A further extension of these studies using the more selective p38 MAPK inhibitor SB 242235 demonstrated that this effect was not only apparent when the compound was administered in a prophylactic dosing regimen, prior to the establishment of lesion, but also using a more clinically relevant therapeutic treatment regimen once the lesion had become established (Badger et al. 2000). This study further highlighted the effect of such treatment in preventing the loss of joint integrity using measurements of bone mineral density, histology, and magnetic resonance imaging. The effectiveness of p38 MAPK inhibition is not limited to the AA rat model. Studies by Aventis Pharmaceuticals have shown the p38 MAPK inhibitors RPR200765A and RPR238677 to also be effective anti-inflammatory and joint protective agents in the streptococcal cell wall-induced arthritis in the rat (McLay et al. 2001; McKenna et al. 2002). Studies using the rat model of collagen-induced arthritis (CIA) have also demonstrated a significant anti-inflammatory effect of p38 MAPK inhibitors (Revesz et al. 2000). Most recently, FR167653, a p38 MAPK inhibitor from Fujisawa Pharmaceuticals, was shown to prevent the onset and progression of CIA in the rat. This molecule also inhibited osteoclastogenesis induced by RANK-L and TNF (Nishikawa et al. 2003).

4.2 Pulmonary Disease

As in RA, inflammatory cytokines play a crucial role in the pathogenesis of airways inflammation. Cytokines such as TNF, interferon (IFN)- γ , IL-4, IL-5, and chemokines such as IL-8, regulated upon activation normal T cells expressed and secreted (RANTES) and eotaxin have all been demonstrated to be capable of generating or supporting airways inflammation (Barnes et al. 1998). The generation and signaling of many of these mediators has also been shown to be dependent on the MAPK cascade. It is therefore postulated that inhibition of p38 α MAPK may have a beneficial effect in the treatment of pulmonary diseases including asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF).

4.2.1 In Vitro Data Supporting p38 MAPK Activation in Pulmonary Disease

The infiltration of the lungs by eosinophils is a prominent feature of asthma. These bone marrow-derived granulocytes can promote airway remodeling and tissue damage through the release of cytotoxic proteins and oxygen radicals (Giembycz and Lindsay 1999). The differentiation, recruitment, and activation of these cells result from the activity of an array of cytokines and chemokines including IL-3, IL-5, and eotaxin. Data generated using the p38 MAPK inhibitor SB 202190 demonstrated an inhibitory effect upon eosinophil differentiation, degranulation, and cytokine release, suggesting a role for this pathway in eosinophil effector functions (Adachi et al. 2000). Changes in airway osmolarity have been hypothesized to contribute to exercise-induced bronchoconstriction and the late-phase airway response. Exposure of bronchial epithelial cells to hyperosmolar medium has been shown to induce expression of the neutrophil chemoattractant chemokine IL-8, which is inhibited by SB 203580 (Shapiro and Dinarello 1995; Matsumoto et al. 1998; Hashimoto et al. 1999).

4.2.2 In Vivo Data Supporting p38 MAPK Activation in Pulmonary Disease

Consistent with the findings outlined above from in vitro studies with eosinophils, the second generation p38 MAPK inhibitor SB 239063 induced eosinophil apoptosis and significantly reduced antigen-induced lung eosinophilia in both mice and guinea pigs in vivo (Underwood et al. 2000). In contrast to these findings, studies using L-790070, another p38 MAPK inhibitor, revealed no effect on eosinophil numbers or cytokine levels in bronchoalveolar lavage (BAL) from antigen-sensitized and challenged mice. These studies, though, did demonstrate a reduction in neutrophilia and a reversal in the antigen-induced increase in mucus-secreting cell numbers (Nick et al. 2002). IPF, a member of the heterogeneous group of pulmonary fibroses whose etiology is unknown, is a chronic, progressive, and often fatal disorder. In a murine model of this disease, induced by bleomycin, administration of the p38 MAPK inhibitor FR-167653 prior to induction of fibrosis resulted in an amelioration of the fibrosis and pulmonary cachexia (Matsuoka et al. 2002).

4.3 Neurodegeneration

There is a substantial amount of evidence linking the MAPK pathways JNK and p38 with neuronal cell death and Alzheimer's disease. Although much of this evidence supports JNK as being the primary pathway, studies also point to a role for p38 MAPK in these processes (Harper and LoGrasso 2001). Both p38 α and p38 β 2 MAPK are expressed in the hippocampus and cortex of brain by immunoblotting techniques (Mielke et al. 1999; Mielke and Herdegen 2000). Using an antibody to the phosphorylated form of p38 α MAPK, studies demonstrated its association with neuritic plaques, neuropil threads, and neurofibrillary tangles, all of which are characteristic features of Alzheimer's disease (Hensley et al. 1999).

4.3.1

In Vitro Data Supporting a Role for p38 MAPK in Neurodegeneration

Much of the in vitro evidence for a role for p38 MAPK in neuronal cell death comes from studies with PC12 cells, a rat pheochromocytoma cell line which responds to nerve growth factor (NGF). Withdrawal of NGF from these cells results in apoptosis and a concomitant induction of $p38\alpha$ and JNK MAPK activity. Furthermore, these studies also demonstrated that this cell death could be inhibited by the p38 α MAPK inhibitor PD169316 (Kummer et al. 1997). It is becoming clear from a number of studies that activation of $p38\alpha$ MAPK and its relative role in neuronal cell death is stimulus dependent. The induction of apoptosis by calyculin in cortical cultures could partially be reversed by PD169316, whereas the induction of excitotoxic cell death by N-methyl-daspartate (NMDA) was not (Ko et al. 2000). Furthermore, SB 203580 could not rescue apoptotic rat ganglion neurons deprived of neurotrophins (Maas et al. 1998), whereas in a separate study it did extend the survival of retinal ganglion neurons exposed to NMDA (Kikuchi et al. 2000). Most recently, Legos and colleagues demonstrated that the selective p38 MAPK inhibitor SB 239063 could confer neuroprotection to cultured primary neurons exposed to NMDA for 5 min, but not for 60 min (Legos et al. 2002).

4.3.2 In Vivo Data Supporting p38 MAPK Activation in Neurodegeneration

Only a limited number of studies involving p38 MAPK inhibition in vivo have been described. Despite this, much of the data generated in vivo supports the assertion above that various stresses result in the activation of different pathways with subsequent cell death. For example, the p38 MAPK inhibitors SB 203580 and SB 239063 have been shown to reduce both brain injury and neurological deficits in models of ischemic brain injury (Barone et al. 2001; Piao et al. 2003), whereas SB 203580 demonstrated no protective effects in a murine model of traumatic brain injury (Mori et al. 2002). Most recently, a study in a rat spinal injury model demonstrated that inhibition of p38 α MAPK activity could prevent the delayed progressive degeneration of oligodendrocytes in the injured area, and a recovery in motor function if the cord damage was not complete (Horiuchi et al. 2003).

4.4 Other Indications

The therapeutic potential of $p38\alpha$ MAPK inhibitors is not confined to the therapeutic indications outlined above. Preclinical in vivo data also point to a potential effect of $p38\alpha$ inhibition in indications as diverse as ischemia-reperfusion injury (Barancik et al. 2000; Kawashima et al. 2001; Kobayashi et al. 2002), polymicrobial sepsis (Song et al. 2001), lymphoma (Elenitoba-Johnson et al. 2003), and inflammatory bowel disease (Waetzig et al. 2002).

5 Conclusion

Over the past decade, the p38 MAPKs have been the subject of intense multidisciplinary research. The discovery of potent selective inhibitors of this pathway has led to a greater understanding of their role in signal transduction and response pathways. The role of these enzymes, particularly p38 α MAPK in the generation of pro-inflammatory mediators, including cytokines, PGE₂, and iNOS, has led to an intensive effort among the pharmaceutical industry to identify clinical candidates for the treatment of a variety of inflammatory diseases. Several of these inhibitors have now entered human clinical trials and demonstrated good pharmacokinetic and pharmacodynamic effects. To date no compound has entered phase III trials; however, several have reached the phase II stage. In all studies, reported administration of drug has resulted in an inhibition of either in vivo or ex vivo endotoxin-induced inflammatory cytokine release. Concern over safety issues has stopped many of these studies. A possible reason for these adverse events may be a cross-reactivity of these compounds with other kinases or enzymes. A majority of the p38 MAPK inhibitors described within this article are ATP competitive. One exception is BIRB-796 that binds to the ATP site but not in an ATP-competitive manner. The BIRB-796 series of inhibitors, which are non-competitive for ATP, may offer better selectivity. The interaction of p38 MAPK with TAB1 (Ge et al. 2002) offers the intriguing possibility that disruption of such an association may also have benefits over the ATP-competitive inhibitors. Further research on the role of the other p38 homologs may also offer an alternative approach for intervention in the future. For example, all four isoforms have shown to be activated as a result of myeloid cell exposure to inflammatory stimuli. There is no doubt that the plethora of preclinical data so far amassed for this molecular target certainly warrants further investigations, not only to identify a clinical candidate but also the appropriate therapeutic indication.

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Inhibitors of PKA and Related Protein Kinases

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Abstract The AGC group of protein kinases comprises a number of pharmacologically important members—targets for small molecule inhibitors of therapeutic value. Crystal structure data assist in the design of new or improved inhibitory molecules. Protein kinase A (PKA), one of the longest and best-known members of the AGC kinase group, has

been cocrystallized with many AGC group inhibitors from highly diverse chemical groups, including isoquinoline derivatives, staurosporine and bisindolylmaleimide cognates, and balanol and pyridine derivatives, thus providing structural information about binding modes, selectivity and cross selectivity. The creation of 'ersatz' kinases by mutating the inhibitor binding site of PKA to resemble other fellow kinases from the AGC group and the cocrystallization of these ersatz kinases with small molecules as well as cocrystal structures of other AGC kinases like 3-phosphoinositide-dependent kinase 1 (PDK1) with staurosporine and bisindolylmaleimide derivatives helps in the identification and exploration of factors governing selectivity.

Keywords PKA · PKC · PDK1 · Rho kinase · Isoquinoline · H7 · H8 · H89 · H-1152P · HA-1077 · Fasudil · Y-27632 · Balanol · Staurosporine · UCN01 · Bisindolylmaleimide 2 · LY333531

1 Introduction

The central role of protein kinases in cellular regulation and signalling control is reflected in the frequent connection between failures in kinase control and serious disease, as is the case in the majority of human cancers. Because most protein kinases are active only when signalling, diseases typically arise from excess rather than loss of kinase activity, caused by mutation, overexpression or disabled cellular inhibition. Several protein kinases, in addition, contribute to disease in the course of their normal cellular function in cell survival, tumour vascularization, cell migration or vascular functions. Pharmacological targets include kinases from the group of AGC kinases, both because of excessive activity and also because of 'normal' functions. While the first protein kinase inhibitors, several of them directed against the classical AGC kinases PKA, PKG, and PKC, were used mostly to elucidate the role of protein phosphorylation in signal transduction pathways and cellular regulation, protein kinase inhibitors now have long reached general acceptance as pharmacological tools of proven high therapeutic value (Traxler 2003). The first protein kinase inhibitor with clinical approval was the AGC kinase inhibitor fasudil or HA1077 (see chapter of Hidaka et al. on fasudil in this volume). Of the 518 protein kinases in man, 62 belong to the subgroup of AGC kinases. Pharmacologically important members of the AGC branch include Aurora, PKB/Akt, 3-phosphoinositide-dependent kinase 1 (PDK1), Rho kinase, and the various isoforms of PKC. PKA, PKC and PKG were amongst the first protein kinases to be identified and isolated, and the accumulated research on these enzymes represents a large proportion of our present knowledge about protein kinase function. Most of the biochemical work was performed with PKA, the so-called 'prototype' protein kinase. PKA was isolated in 1968 (Walsh et al. 1968) and has been studied with respect to protein kinase function ever since. PKA is the central effector of cyclic adenosine monophosphate (cAMP), the second messenger involved in a myriad of signalling pathways for metabolism, gene transcription, memory function, ion channel regulation, cell proliferation and cell differentiation (Montminy 1997). PKA also is a marker and a target in malignant disease; antisense inhibitors targeting the RI subunits are in early clinical trials against a variety of tumours (Chen et al. 2000). The activity of the catalytic subunit also appears to be associated with the development of Alzheimer's disease (Marambaud et al. 1999).

PKA is a prototype enzyme for the protein kinase family for three reasons. (1) PKA is quite abundant in tissue, and efficient highly specific purification methods were discovered early (Kinzel and Kubler 1976; Kubler et al. 1979; Nelson and Taylor 1981) to enable biochemical investigation and also to provide highly homogeneous enzyme for subsequent crystallographic studies (Bossemeyer et al. 1993). (2) The catalytic subunit forms an inactive holoenzyme in the absence of cAMP, consisting of catalytic and regulatory subunits, but is monomeric in the presence of high cAMP concentrations. It consists almost entirely of the conserved catalytic core of protein kinases, constituted by 245 out of 350 residues of PKA (Hanks and Quinn 1991). (3) The monomeric C-subunit is always found phosphorylated at its activation loop threonine residue and is thus in an active state. Together, these circumstances make PKA ideally suited for studies on substrate recognition, catalytic pathway, order of substrate and cofactor binding, the identification of catalytic site residues, the cofactor specificity and its interaction with pseudosubstrate inhibitors, such as the protein kinase inhibitor and the R-subunits. Consequently, the first crystal structure of a protein kinase was from the catalytic subunit of PKA (Knighton et al. 1991).

Most known protein kinase inhibitors act in competition to ATP, binding in the ATP binding pocket, and mimicking many of the ATP enzyme interactions. The crystal structures of PKA in complex with MgATP or MnAMP-PNP and a pseudosubstrate peptide (Zheng et al. 1993; Bossemeyer et al. 1993) showed in detail the binding of the ATP molecule to protein kinases. These structures showed the functions to most of the residues, which are invariantly conserved in the kinase family. Together with the vast amount of previous data from biochemical and genetic studies, the structures explained in detail most principles of the catalytic mechanism of protein kinases, recently further confirmed by the structure of the putative transition state of the phosphorylation reaction (Madhusudan et al. 2002). In addition to the unique structural properties of the kinase-conserved glycine-rich loop, the structural origins of the synergistic binding of cofactor and pseudosubstrate inhibitors became apparent (Bossemeyer 1994). In this way, these early structures provided the first insights into the binding site relevant to the majority of all protein kinase inhibitors.

1.1 Specificity Features of the ATP Binding Site

The ATP binding site of protein kinases consists of two subsites with very different properties: the triphosphoryl subsite contains most of the invariantly conserved residues and is thus entirely conserved. All residues with sidechain contacts to the ATP molecule are either charged or electrophilic. The contacts of the triphosphoryl group to the enzyme form an extensive electrophilic network that involves the conserved sidechains either directly or via metal ions, and also involves several backbone atoms from the glycine-rich loop. Both the interactions with conserved sidechains and the loop interactions, because of the high structural homology of the protein kinases, seem likely to be conserved. Structures from other protein kinases with ATP or ATP analogues, however, showed that these contacts are not always identical. This is especially true for the metal ions, and the contacts to the glycine-rich loop (Russo et al. 1996; Hubbard 1997; Aubol et al. 2003; Nolen et al. 2003). Apparently, although at least within each of the subfamilies of the Ser/Thr and the Tyr-specific kinases amino acid residues in contact with the triphosphoryl group are strictly conserved, structural or electrical differences appear to exist and thus might contribute to the selectivity of suitable kinase inhibitors. In contrast to this highly conserved triphosphoryl subsite, the adenosine binding site contains only one invariant residue, the homologue of Val57, located at the C-terminus of the glycine-rich motif in PKA, and one very conserved residue, the homologue of Ala70 from β -strand 3. All other residues with sidechain contacts to ATP are variable but conservatively exchanged in the kinase family. Charged residues exist only in the ribose-hydroxyl subsite, Glu (corresponding to Glu127 of PKA), and Asp residues are common here. Apart from that, few residues are hydrophilic, such as Thr183 (PKA) and most are hydrophobic. The conserved polar interactions of adenosine in the binding site are again with backbone atoms, in PKA from N1 of the adenosine moiety to the amide of Val123 and from N6 to the carbonyl of Glu121. A functional reason for the variability of residues in the ATP site is not obvious. As all kinases bind ATP, differences might affect ATP binding and adenosine diphosphate (ADP) release rates, and could conceivably affect catalytic rates as well. As kinase (down)regulation often is accompanied by significant structural changes (Engh and Bossemeyer 2001; Huse and Kuriyan 2002), a specific role of ATP-site residues in inactivated conformations might also exist. A correspondence between the rotamer conformation of a residue on the enzyme surface and the sidechain change of a residue in the adenosine binding site has been shown recently with mutants of PKA. Gln181, usually in sidechain contact with hydrophilic residues on the surface, changed its conformation and partly obstructed the adenosine binding site after exchange of Val123, a variable hinge region residue, to alanine (Gaßel et al. 2003). As will be discussed in more detail later, a corresponding conformational change of such a Gln residue has been observed in the structure of PDK1 with UCN01 (Komander et al. 2003). PDK1 has alanine in the Val123 (PKA) position too. Exchange of Gln181 in PKA to the much more conserved lysine in the position of Gln181 resulted in a fixed surface orientation (Gaßel et al. 2003). This clearly demonstrates secondary functional consequences of single residue exchanges that require compensation of some sort. Thus, the high variability of residues which line the adenosine subsite may represent functional differences or may simply reflect less stringent evolutionary constraints in this region; in either case, it offers interesting opportunities to selectively target protein kinases with small molecule inhibitors by making use of the small differences in the individual shape and electronic environment in each protein kinase ATP pocket.

The sequence similarity of kinases within the same branch of kinases, such as the AGC kinases, leads to cross selectivity of protein kinase inhibitors. Obviously, closely related kinases appear to have a higher conservation of the kinase fold. A good example is the structures of active PKB (Yang et al. 2002), showing an ATP binding site which is almost indistinguishable from that of PKA. Mutagenesis of PKA and exchange of residues in the ATP pocket to PKB-specific residues emphasizes these similarities even further (Gaßel et al. 2003). Correspondingly, commercially available protein kinase inhibitors have very similar activities against both kinases (Davies et al. 2000). This is true for several protein kinase inhibitors that target AGC kinases. Though certainly a predicament for the design and development of selective protein kinase inhibitors, it can be turned into an advantage to achieve structural information about inhibitor/target interactions even if the primary target is difficult to deal with. PKA has been used as an ersatz kinase to study the binding mode of various AGC kinase inhibitors, mostly in cases were the actual target was structurally not available. For a long time PKA was the only AGC kinase with a known crystal structure. In the last 2 years, the crystal structures of several other members of the AGC branch have been solved, such as PKB/Akt (Yang et al. 2002), PDK1 (Biondi et al. 2002), Aurora (Bayliss et al. 2003) and Grk2 (Lodowski et al. 2003).

2 Inhibitor Cocrystal Structures

2.1

Structures of PKA with Isoquinoline Sulphonamide Derivatives

PKA was among the first kinases cocrystallized with protein kinase inhibitors (Schulze Gahmen et al. 1995; De Azevedo et al. 1996; Engh et al. 1996; Xu et al. 1996). The isoquinoline sulphonamide derivatives H7 (1-(5isoquinolinesulfonyl)-2-methylpiperazine), H8 (N-[2-(methylamino)-ethyl]- 5-isoquinolinesulphonamide), and H89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide) were chosen, because these inhibitors were at that time in wide use, and all of them inhibit PKA (PDB-codes 1YDR, 1YDS, 1YDT). H7 has a broad specificity within the AGC kinases and inhibits PKC as well as PKA in the micromolar range. Its ability to inhibit PKC (at 6 μ M) was the reason for the enormous popularity of H7, used by many laboratories to dissect the role of PKC in signal transduction. Rho kinase, actually, is a 20-fold better target for H7 (Uehata et al. 1997) (300 nM), but this enzyme was not described before 1995 (Leung et al. 1995). H8, a micromolar inhibitor of PKA and PKC, inhibits PKG also. Still, H89 (48 nM for PKA) is one of the more selective PKA inhibitors, although when tested against a wider panel of protein kinases, some other AGC kinases, such as Rho kinase and PKB/Akt from the AGC group and S6K1 and MSK1 from other branches of the kinase family, are found to be inhibited by H89 too at comparable concentrations.

The H-inhibitors are characterized by an isoquinoline sulphonamide group, and a sidechain, where a two-carbon spacer separates two amide groups.

The binding of H7 to PKA is representative for some typical aspects of PKA inhibitor binding. The conserved isoquinoline moiety of the inhibitor, a planar double ring with one nitrogen atom acting as proton acceptor, occupies the position of the adenine purine of ATP and mimics the ATP N1 H-bond to the hinge region Val123 amide (Fig. 1). The planar isoquinoline group is embedded between the residues Val57, Ala70 and Leu49 from the small lobe, and Leu173 from the large lobe; these interactions contribute significantly to the number of van der Waals (VDW) contacts to the enzyme. Usually protein kinase inhibitors bind alike, with a proton accepting interaction from a planar structure. Few structures are known where the inhibitor does not follow this pattern. TBB (tetrabromo-2-benzotriazole) (PDB-code 1J91) is such an example, which makes no hinge atom contact in CK2 (Battistutta et al. 2001); however, this appears to be a specific feature of CK2,

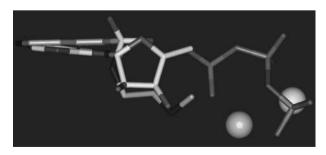


Fig. 1 Superimposition of H8 and MnAMP-PNP, showing spatial congruence with adenosine group and of function groups. (Engh et al. 1996)

because the same inhibitor binds in CDK2 according to the common pattern (De Moliner et al. 2003). Also the inhibitor BIRB 796 (PDB-code 1KV1) binds to a 'DFG-out' conformation of P38 in an atypical way without a hinge region contact (Pargellis et al. 2002). In many AGC kinases, such as PKA, or PKB, a residue from outside the catalytic core, Phe327, contributes to the completeness of the adenosine binding site. Phe327 or its homologue is positioned on a C-terminal polypeptide stretch that expands from the large lobe via the catalytic cleft to the N-terminal lobe and ends in the hydrophobic motif beside helix C. This aromatic residue is not conserved outside the group of AGC kinases, nor has a comparable contribution been observed in other kinases so far. The H-inhibitors mimic not only the purine of ATP, but also aspects of the ribose. The 2' and 3' OH groups of ATP in PKA make contacts to the backbone carbonyl of Glu170, and in addition to the sidechain of Glu127, the only charged residue in the adenosine pocket. The sulphonamide group together with the sidechain mimics a part of the ribose spatially, and the distal amine of the aminoethyl sidechain of most H-inhibitors forms a similar contact to the Glu170 carbonyl. Interestingly, one of the sulphonyl oxygens is in the same spatial position as the ring oxygen of the ribose. This is not only observed with H7, but is a general feature of H-inhibitor binding in PKA, and is observed also with the indolocarbazole staurosporine. In none of these cases is a typical H-bond contact formed from the oxygen, but the C α -hydrogen of Gly50 is close enough for a weak CH-O interaction, as was postulated for staurosporine (Zhu et al. 1999). The triphosphoryl subsite, however, is not occupied by most of the PKA inhibitors, with the exception of balanol (see below), which makes electrophilic contacts to Lys72 (in its normal function involved in binding the phosphoryl groups) and the backbone amides of the glycine loop. The consequence is that, in the case of H7 and H8, the glycine-rich flap is not in contact with the inhibitors and has an increased mobility. This is different in the case of H89, which possesses a large bromocinnamyl sidechain, binding underneath and stabilizing the structure of the glycine flap (Fig. 2). The specificity of the H-inhibitors is defined by differences in their sulphonamide substituents, as all (with the exception of H-1152P) have identical isoquinoline sulphonamide head groups. Accordingly, the number of VDW contacts to the isoquinoline is almost identical for all three inhibitors, only H89 is packed slightly better. Differences are observed for the contacts of the amino group of the sidechains, although this group is present in all three inhibitors. Only H8 makes a contact from here to the sidechain of Asp184. Asp184 usually has a different rotamer and is in contact with the basic charge of Lys72, or, in the ATP-bound structure, with one of the metal ions. Apparently, the distal amine in the H8 sidechain attracts this residue more than the comparable nitrogens in H7 or H89, which even is structurally identical to H8 in this region. The bromocinnamoyl group and the piperazine ring weaken the partial-positive charge of the secondary amine, which

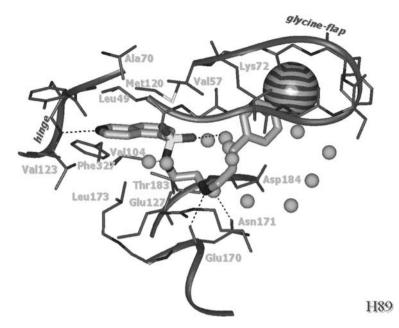


Fig. 2 H89 in the binding pocket of PKA. The bromocinnamoyl group interacts with the glycine-rich motif. (Engh et al. 1996)

then is less attractive for the Asp residue and does not induce a conformational change. The overall number of polar and VDW interactions of these inhibitors varies; H89 has three H-bond contacts to the enzyme and the highest number of VDW interactions. A correlation of structural features to the inhibitory activity was found in the total area of the buried surface of the inhibitor (Engh and Bossemeyer 2002). Inhibitor selectivity in closely related kinases is determined mostly by sidechain differences in the ATP pocket. One PKA related kinase, hardly inhibited by H89, is PDK1. Few residues differ in the ATP binding pocket between PDK1 and PKA: Val123 is Ala in PDK1, Met120 is Leu in PDK1; both residues, however, interact with the isoquinoline head group, and not with the H89-specific sidechain. A significant difference in a sidechain-critical region is Gly55, the first residue following the turn in the glycine-rich beta sheet. This residue is Ser in PDK1. Interestingly, Phky, although also quite similar to PKA in the binding pocket, is also much more weakly inhibited by H89 and also contains a serine residue in the Gly55 position. Another general difference between PKA and PDK1 is the absence of the C-terminal stretch with the phenylalanine residue which, as Phe327, makes contacts to the isoquinoline of all H-inhibitors. Perhaps the binding pocket for comparably small molecules such as H-inhibitors often requires some completeness, provided for most AGC kinases by this C-terminal phenylalanine residue. It cannot be excluded that this residue is

one reason for the general preference of several H-inhibitors, such as H89, or HA1077 for AGC kinase, such as MSK1 or S6kinase (Davies et al. 2000). Several derivatives of the isoquinoline sulphonamides have been made which are active against protein kinases. A cognate of H7, HA-1077 or fasudil, was especially successful as an inhibitor of Rho kinase (see the chapter on fasudil by Hidaka et al., this volume). Fasudil and two other Rho kinase-selective inhibitors, H-1152P and Y-27632, were cocrystallized with PKA (Breitenlechner et al. 2003).

2.2

PKA as a Model for Rho Kinase: Structures of PKA with Rho Kinase Inhibitors Y-27632, Fasudil (HA-1077) and H-1152P

As a serine-threonine kinase of the AGC group, Rho kinase possesses a catalytic domain closely related to other AGC group kinases, among them PKA, PKB, PKC and PKG; although no crystal structure of Rho kinase has been reported yet. The close relationship between PKA and Rho kinase (37% identical) and the well-established crystallization conditions for PKA make PKA a suitable model system for studying Rho kinase inhibitors. Furthermore, cocrystallization of PKA with Rho kinase inhibitors helps identify factors governing cross selectivity of protein kinase inhibitors. The importance of sidechain differences in the ATP binding pocket for inhibitor selectivity has been shown previously with Erk2 (Fox et al. 1998).

To avoid confusion, we will use the term 'Rho kinase' for the two enzymes of this family: Rho-associated, coiled-coil-containing protein kinase p160ROCK (gene: *ROCK1*) and Rho kinase (gene: *ROCK2*). They are highly homologous in the kinase domain (96%), especially in ATP pocket and are identical in all residues in contact with the inhibitors.

2.2.1

Inhibitor Binding Site—ATP pocket

The inhibitors are ATP competitive and bind to the ATP pocket made up of residues from the glycine loop (Leu49, Gly50, Thr51, Val57), β -sheet 3 (Ala70), hinge region (Met120, Glu121, Tyr122, Val123, Glu127), catalytic loop (Glu170, Asn171, Leu173), beginning of the activation loop (Thr183, Asp184), and C-terminal stretch (Phe327) (Fig. 3).

The buried surface of the inhibitors in their pocket in PKA correlates with their affinity: H-1152P (1.1 μ M) with the highest affinity also has the largest buried surface of the three inhibitors (215.7 Å²); Y-27632 (25 μ M) has the smallest buried surface of the three (188 Å²). A correlation of buried inhibitor surface and binding affinity has previously been observed for PKA inhibitors (Engh and Bossemeyer 2002). Rho kinase and PKA differ, however, in eight positions in the ATP binding pocket. Four of their sidechains are

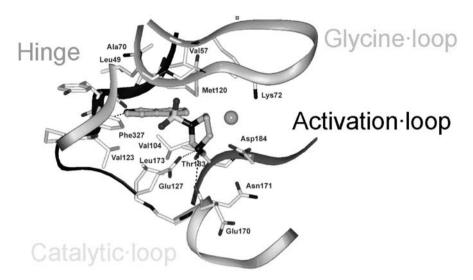


Fig. 3 Binding of HA-1077 (fasudil) in the ATP pocket (Breitenlechner et al. 2003). Hydrogen bonds between enzyme and inhibitor are depicted by *dotted lines*; conserved peptide strands of the kinase domain are shown as *ribbons*

in close (<4 Å) contact with the inhibitors, corresponding to the following PKA \rightarrow Rho substitutions: Leu49Ile, Val123Met, Thr183Ala and Glu127Asp. Because the protein kinase fold is so highly conserved, variations of amino acid residues that line the ATP subsite belong to the most important factors in defining inhibitor selectivity.

2.2.2 Fasudil (HA-1077 or AT877)

Fasudil was the first protein kinase inhibitor approved for clinical use (1995). It is effective—with very little side effects—for cerebral vasospasm, the painful and sometimes deadly after-effect of subarachnoid haemorrhage. Fasudil has significant vasodilatory activity (Ono-Saito et al. 1999) and is now in clinical trials for the treatment of angina pectoris (Shimokawa et al. 2001). Fasudil's activity has been attributed to inhibition of Rho kinase (Matsui et al. 1996) and its role in signalling for myosin light chain phosphorylation and arterial smooth muscle contraction (Amano et al. 1996; Takemoto et al. 2002), although the in vitro activity of fasudil is not strictly limited to Rho kinase. Other Rho kinase-related protein kinases, such as PKA, PRK2, MSK1 and S6K1, are also inhibited by fasudil, although to a lesser extent (Davies et al. 2000). Rho kinase may be an important pharmacological target also for cancer because of its role in the invasion and migration of cancer cells (Amano et al. 2000; Fukata et al. 2001).

Inhibitor	Resolution (Å)	Chemical structure	<i>K</i> _i [μM] (PKA) ^a	K _i [μM] (Rho kinase) ^a
HA-1077	2.2		1	0.33
		0 10 10 10 11 11 10 10 10 10 9 9 9 9 9 9		
H-1152P	1.9	⁴ ⁵ ⁶ ⁷ ¹⁰ ¹⁰	0.63	0.0016
		Me O = 0 = 0 = 0 = 0 = 0 = 0 = 0 = 0 = 0 =		
Y-27632	2.0	10		0.14
	HA-1077 H-1152P	(Å) HA-1077 2.2 H-1152P 1.9	(Å) HA-1077 2.2 H_{1}^{-1} H_{2}^{-1}	HA-1077 2.2 H 1 HA-1077 2.2 H 1 H 1 H-1152P 1.9 H 0.63 Y-27632 2.0 f 25 Y-27632 2.0 f 25

 Table 1
 Crystal structures of PKA-Rho kinase inhibitor complexes

^a Ikenoya et al. 2002.

Fasudil is related to H7, and has a heptameric homopiperazine ring at the position of the methyl-piperazine ring of H7 (Table 1).

The binding of fasudil is similar to the binding of the parental molecule H7 to PKA (Engh et al. 1996) (Fig. 3). The essential hydrogen bond between the peptide backbone amide of Val123 and the nitrogen atom of the isoquinoline ring is formed. As H7, the secondary amine of the homopiperazine ring makes a hydrogen contact to the peptide backbone carbonyl of Glu170. This contact, usually reserved for the 3' OH of ATP, is present in the PKA/H7 structure too. Not provided by H7, fasudil can make, in addition, a contact of the homopiperazine nitrogen to the sidechain of Glu127. In the ATP structure this contact is also present, to the 2' and 3' OH groups of the ribose.

2.2.3 H-1152P

Further derivatization of fasudil led to H-1152P, with two additional methyl groups, one at the isoquinoline ring and the other at the homopiperazine ring (Tanaka et al. 1998). H-1152P inhibits Rho kinase in the low nanomolar range, with a 400-fold selectivity over PKA (Sasaki et al. 2002). Fasudil and H-1152P bind both with similar affinity to PKA in the same orientation in the adenosine pocket (Fig. 4). H-1152P, however, has only one H-bond contact to the enzyme, the one between the hinge region amide group to the isoquinoline ring nitrogen. No H-bonds are formed with the nitrogen atom of the homopiperazine ring. The reason for this difference in binding of H-1152P and fasudil to PKA becomes obvious from an overlay of both inhibitor structures. In order to form the 'essential' H-bond to the hinge region, H-1152P can only bind, when the distal (as seen from the hinge) homopiperazine ring is shifted toward the glycine-rich loop in order to avoid a steric clash between the extra methyl group at the homopiperazine ring, and the sidechain of Thr183.

Thr183 is one of the four residues which are in sidechain contact to ATPside inhibitors and differ between PKA and Rho kinase. An alanine residue in this position, as in Rho kinase, would provide enough space for a closer

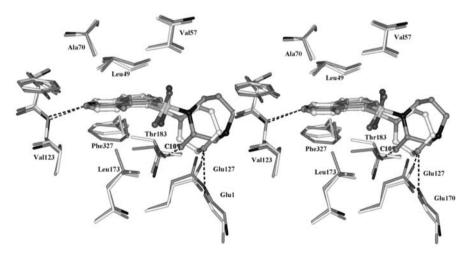


Fig. 4 Comparison of HA-1077 and H-1152P. Superimposed are the inhibitor binding sites of fasudil and H-1152P in PKA. A *double arrow* indicates a possible steric conflict between the selectivity defining methyl group C10 of H-1152P and the sidechain of Thr183, a residue which is alanine in Rho kinase. (Breitenlechner et al. 2003)

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approach of the H-1152P homopiperazine ring towards the H-bond-forming groups of Glu170, and Gly127 (Fig. 4). Also, the exchange of the Leu49 residue to isoleucine could be an advantage for H-1152P, by increasing the number of VDW contacts and the hydrophobic interactions. The extra methyl group at the isoquinoline ring could benefit from the branching of the isoleucine sidechain at the C β atom. The two other differences, Val123Met and Glu127Asp, are difficult to interpret in terms of selectivity for Rho kinase. The valine residue makes the largest number of contacts with fasudil, and the second largest number of contacts with H-1152P. As seen in protein kinase crystal structures with a methionine residue in the homologous position, the sidechain points away from the ATP binding pocket such that only $C\beta$ or $C\gamma$ atoms can interact with an ATP-site ligand. The methionine therefore does not likely contribute to Rho kinase selectivity for HA-1077 or H-1152P, since its effect, if any, would be to remove interactions and lower the binding affinity (S. Bonn, S. Herrero, M. Gaßel, C.B. Breitenlechner, R. Engh, D. Bossemeyer, in preparation). An exchange of Glu127 to Asp is also expected to change little, because the shorter sidechain can still make the H-bond contacts to the homopiperazine nitrogen atoms. Overall, the contacts from the residues which differ between PKA and Rho kinase in the ATP binding site contribute most significantly to the interactions between the inhibitors and PKA.

Because sidechain interactions form the major kinase selectivity determinant of active conformations to ATP-site inhibitors, the conservation of Thr183, Val123, Leu49 and Glu127 should be predictive of H-1152P selectivity. None of these four residues is rare or exceptional (Table 2). However, this combination occurs in only six other kinases, none of which belong to the AGC group of protein kinases. Consideration of a fifth residue renders Rho kinases unique. AGC kinases, including PKA and Rho kinase, possess a C-terminal strand that inserts a phenylalanine sidechain in to the ATP binding site. The phenylalanine interacts in known AGC kinase structures with the purine base of ATP and most ATP-site ligands, and also with fasudil and H-1152P. The ATP-site ligand contacts to Phe327 are typically edge-edge aromat aromat contacts that affix the ligand to the adenine site. Thus, the

РКА	Residue number	Rho-kinase	Occurrence (%)
Thr	183	Ala	34/29
Val	123	Met	29/25
Leu	49	Ile	44/38
Glu	127	Asp	38/32

Table 2 Differences in Rho kinase and PKA and occurrence (according to Hanks and Quinn1991; Manning et al. 2002)

combination of Thr183, Val123, Leu49 and Glu127 together with Phe327 is unique to Rho kinase.

2.2.4 Second Binding Site for H-1152P

In addition, for H-1152P a second binding site exists in a surface region with contact to the phosphoryl group of Thr197 and to Lys189 (Fig. 5), both from the activation loop and to Glu86 from helix C, a region critical for kinase activity and protein–protein interaction with the regulatory R-subunit (Gibbs et al. 1992; Orellana et al. 1993). Although a functional relevance of H-1152P

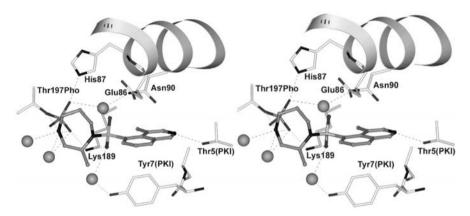


Fig. 5 H-1152P binds in a second position on the surface of the protein in contact with activation loop phospho-Thr197, and helix C

in this second position for PKA is not clear yet, this site might be attractive as a new target site for drugs that interfere with kinase regulatory elements or regulatory subunits.

2.2.5 Y-27632

Y-27632, a pyridine derivative, represents a chemical class distinct from the H inhibitors described above. It has a K_i of 140 nM for Rho kinase and 25 μ M for PKA (Table 1) (Ishizaki et al. 2000) and is ATP competitive, like the H inhibitors (Ikenoya et al. 2002; Trauger et al. 2002). Y-27623 is a widely used Rho kinase inhibitor and several effects could be observed in vitro and in vivo (for review see Ishizaki et al. 2000; Narumiya et al. 2000). It prevents phosphorylation of focal adhesion kinase and paxillin (Sinnett-Smith et al. 2001), inhibits the migration of rat MM1 hepatoma cancer cells (Itoh et al.

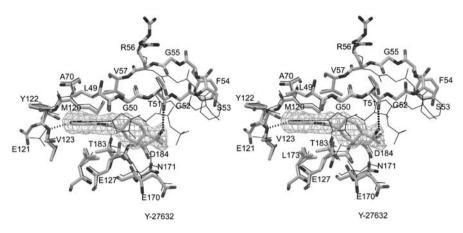


Fig. 6 Binding of Y-27632 to PKA to the ATP binding site. Electron density is displayed at 2σ . (Breitenlechner et al. 2003)

1999), and leads to smooth muscle contraction and normalization of blood pressure in rats (Uehata et al. 1997). A pyridine ring is connected by an amide to a saturated para-aminoethyl cyclohexane ring. The pyridine similarly occupies the adenine subsite and with the canonical H-bond contacts to the hinge region the Val123 amide group (Fig. 6). The cyclohexane is situated in the ribose subsite. A hydrogen bond is formed from the terminal aminoethyl group to the carbonyl oxygen of Thr51. As the cyclohexane moiety can bind in two slightly different orientations, alternatively, hydrogen bonds from the terminal aminoethyl group to the sidechains of Asp171 and Asp184 are possible too.

The selectivity of Y-27632 for Rho kinase over PKA is 200-fold, a property not immediately rationalized by the structure. It seems, however, likely that the Leu49Ile exchange can enhance hydrophobic interactions. At the same time, the exchange Thr183Ala in Rho kinase offers a wider pocket and greater freedom for the inhibitor to optimize binding interactions.

2.3 Balanol and Derivatives

The balanols represent a class of potentially therapeutic PKC inhibitors. They are derived from the natural product (–)-balanol, which was first isolated as a metabolite of the fungus *Verticillium balanoides* at Sphinx Pharmaceuticals (Eli Lilly) (Kulanthaivel et al. 1993) and from a species of *Fusarium* at Nippon Roche (Ohshima et al. 1994). (–)-Balanol inhibits PKC and PKA in the low nanomolar range (K_i =6.4 nM for PKC α , K_i =1.8 nM for PKC β II and K_i =3.9 nM for PKA) and other serine/threonine kinases less potently (e.g. 74 nM for CaMKII and 742 for MAPK); tyrosine kinases are not inhibited (Setyawan et al. 1999). A cocrystal structure of (–)-balanol with PKA was published in 1999 (1BX6) (Hunenberger et al. 1999; Narayana et al. 1999). Balanol derivatives with PKC inhibitory activity have been reported in the literature (Hall et al. 1994; Barbier et al. 1995; Jagdmann et al. 1996; Gustafsson and Brunton 1999; Setyawan et al. 1999; Lampe et al. 2002). New derivatives of balanol have been reported as protein kinase B (PKB/Akt) inhibitors. Cocrystal structures reveal binding modes and are instrumental in optimization of properties, such as selectivity profile against PKA or PKC, etc. (Breitenlechner et al. 2004).

2.3.1

X-Ray Structure of Balanol (PDB-code 1BX6)

The complex of the natural (-)-balanol with PKA (PDB-code 1BX6) was solved in 1999 (Narayana et al. 1999). The molecule (Fig. 7) can be considered in terms of occupation of three regions of the ATP binding pocket: a phenol (Fig. 7, ring A) in the adenine subsite, an azepane (B) in the ribose subsite and the benzophenone (C and D) occupies the triphosphate subsite.

The phenol makes two H-bonds from the -OH group to the hinge region Val123(N) and Glu121(O); VDW contacts exist to Leu49, Ala70, Glu121, Tyr122, Val123, Thr183 and Phe327. The amide that links to the azepane ring makes one H-bond to a water molecule with its amide nitrogen and to Thr183 with its carbonyl oxygen. The azepane ring is in VDW contact with Gly50, Glu127 and Glu170, and additionally forms an H-bond with Gly170. The benzophenone part interacts with Thr51, Gly52, Ser53, Phe54, Gly55, Arg56, Lys72, Leu74, Gln84, Glu91, Asp184, Gly186, Phe187. Benzophenone substituents are involved in several H-bonds: to Gly52, Ser53, Phe54, and

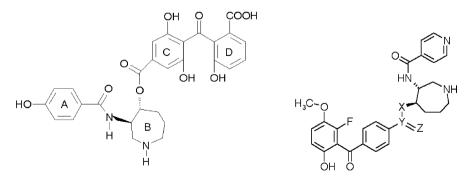


Fig. 7 Chemical structure of the natural (–)-balanol and the novel balanol derivatives. (Breitenlechner et al. 2004)

Gly55 from the glycine loop, to the conserved residues Lys72 and Glu91, and to Asp184 from the beginning of the activation loop.

In total, there are 21 H-bonds and 114 VDW interactions, whereas most occur with the triphosphate subsite (Narayana et al. 1999; Setyawan et al. 1999; Wong et al. 2001).

2.3.2 X-Ray Structures and Binding Mode of Balanol Derivatives

Novel azepane derivatives have been synthesized and the X-ray structures of the PKA-PKI-complexes solved (Breitenlechner et al. 2004). The chemical structures involve variations of different parts of the natural balanol: the phenol has been replaced by a pyridine ring, the substituents on the benzophenone have been modified and the ester linker between ring B and C has been extensively modified in order to optimize pharmacokinetic properties.

Three binding regions were defined in reference to the balanol-type interactions: the pyridine pocket, the azepane pocket and the benzophenone pocket (Fig. 8). These regions correspond closely to the adenine, ribose and phosphate binding sites (Breitenlechner et al. 2004).

The pyridine pocket contains the pyridine moiety and the amide connecting it to the azepane ring. This space is occupied by the adenine in the complex of AMP-PNP and PKA (Bossemeyer et al. 1993) (Fig. 8) and accepts in general aromatic groups. The glycine loop (Leu49, Val57), β -sheet 3 (Ala70), the hinge region (Met120, Tyr122, Val123, Glu127), the catalytic loop (Leu173), the beginning of the activation loop (Thr183), and Phe327 from the C-terminal stretch are involved in VDW contacts to the pyridine and amide portion of compound 1 (Fig. 9).

The hinge region residue Val123 makes an H-bond to the pyridine via its main chain amide. This H-bond to Val123 (or its homologue in other kinases) is nearly universal amongst protein kinase inhibitor complexes and is

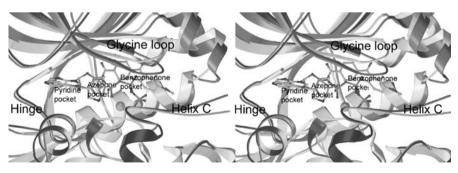


Fig. 8 Binding of a balanol derivative to the ATP binding pocket of PKA (*light grey*) superimposed with the AMP-PNP structure (*dark grey*); [1CDK (Bossemeyer et al. 1993)]

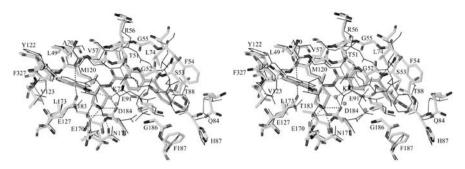


Fig. 9 Binding of a balanol derivative to the ATP binding pocket of PKA in structural detail

apparently critical for tight-binding inhibitors. Two H-bonds exist from the amide group to two water molecules that bridge polar contacts between the amide NH and Glu127 and Leu49 and between the amides CO to Lys72 and Asp184.

The azepane pocket—stretching over the ribose subsite—contains the azepane and the ester part of the molecule. This pocket is bounded by residues from the glycine loop (Gly50, Thr51, Gly52, Val57), β -sheet 3 (Lys72), the catalytic loop (Glu170, Asn171) and Asp184 from the beginning of the activation loop. The protonated azepane nitrogen forms H-bonds to Asn171 and Asp184.

The benzophenone pocket is bounded on one side by the flexible glycine loop, which is displaced compared to the AMP-PNP PKA complex (see Fig. 8). Consequently, VDW contacts exist to glycine loop residues (Ser53, Phe54, Gly55, Arg56), and also to residue Leu74 from the β -sheet 3, to several residues from helix C (Gln84, His87, Thr88, Glu91), as well as to Gly186 and Phe187 from the activation loop.

The carbonyl oxygen of the benzophenone is involved in hydrogen bonds to the backbone amide nitrogen Phe54 at the tip of the glycine loop.

Glu91, which is essential for catalysis (Schneider 2002), forms a salt bridge to Lys72 in the active state as well as in the inhibitor complex with compound 1. In this complex, Glu91 can also form a hydrogen bond to the inhibitor hydroxyl group of the benzophenone (Breitenlechner et al. 2004).

2.3.3

Binding Affinity and Mode of Binding of Related Balanol Derivatives

The other inhibitors 4, 5, and 8, with amide, ether, and vinyl linkers between the azepane and benzophenone moieties, were cocrystallized with PKA; the structures are superimposed with the ester 1 in Fig. 10. The crystal structures confirm that the bound conformations are unperturbed by the modifications. The conformation of the linker for which the torsional angle is *an*-

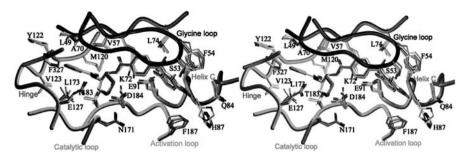


Fig. 10 Intermolecular bonding contacts in the complex between PKA and the balanol derivative. (Breitenlechner et al. 2004)

tiperiplanar in all molecules is *trans* with respect to azepane and benzophenone. This conformation is required because it orients the azepane and the benzophenone towards their binding pockets. Therefore, molecules which are preorganized in aqueous solution in the *trans* conformations should have higher binding affinities [which is in agreement with the biochemical data (Table 3)]. And indeed, ester 1 and amide 4 show tight binding (IC₅₀=5 nM or 4 nM, respectively), similar to (–)-balanol.

In the complex between the double amide 4 and PKA, there is a rearrangement of the sidechains of Asp184, Thr183 and Lys72 compared to the complex of the ester with PKA. The sidechain of Asp184 is rotated towards the amide linker and makes an H-bond to the amide nitrogen in addition to the H-bond to the azepane nitrogen; Lys72 forms an additional H-bond to the inhibitor hydroxyl group of the benzophenone, and Thr183 H-bonds to

Inhibitor	X-YZ	Linker	Half-life ^a	IC ₅₀ (PKB) ^a	IC ₅₀ (PKA) ^a
1	O-C=O	Ester	<1 min	5 nM	5 nM
4	NH-C=O	Amide	69 h	4 nM	2 nM
5	O-CH ₂	Ether	29 h	355 nM	39 nM
8	CH=CH	Vinyl		160 nM	360 nM

Table 3 Properties of balanol derivatives

^a Breitenlechner et al. 2004.

the amide next to the pyridine. In the cocrystal structure of the ether and vinyl compounds, the orientation of Asp184 corresponds to the complex of the ester with PKA, whereas the conformation of the sidechains of Thr183 and Lys72 are similar to the amide cocrystal structure (Breitenlechner et al. 2004).

2.3.4 Comparison to the (–)-Balanol

The binding mode of (-)-balanol and the new derivatives is basically the same, although there are some distinct differences. The phenol and pyridine share the canonical H-bond to the hinge region residue Val123. Additionally, the phenol can make a second H-bond to the carbonyl of Tyr122. The benzophenone in (-)-balanol has two hydroxyl groups on ring C and one hydroxyl and one carboxylate on ring D. These highly hydrophilic substituents are involved in extensive H-bonds (see above). The derivatives have substituents only on ring D, the hydroxyl group, a fluor substituent in the position of the carboxylate group and an additional methoxy group. The fluor substituent points in a similar direction as the carboxylate (towards Ser53), but is unable to make an H-bond. The hydroxyl substituent has hydrophilic contacts or H-bonds to Glu91 and Lys72 that are similar in all structures. The methoxy group extends deeper into another pocket, towards Phe187, Gln84 and His87, which is not occupied by the (-)-balanol. Asp184 of the conserved DFG motif that initiates the activation loop deserves special attention. It is probably the most flexible residue in the ATP pocket, with the greatest degree of ligand-induced motions: its sidechain points away from the pocket when it coordinates with the manganese atoms in the AMP-PNP structure (PDB-code 1CDK), or the hydroxyl group on the benzophenone ring (C) in the (-)-balanol complex, and also in the presence of ligands such as H-1152P, Y-27632, H89 and H7, but it points into the pocket with ligands such as H8 or HA-1077 and the azepane derivatives. In the latter case it forms an H-bond to the azepane nitrogen, except for the double amide 4, where the H-bond is made to the amide nitrogen of the linker. Thr183 and Leu173 seem also to be flexible, and their sidechain orientation varies. Thus the hydroxyl group of Thr183 makes H-bonds either to a conserved water molecule or to the inhibitor.

2.4 Structure of PKA with Staurosporine

Amongst the potentially therapeutic inhibitors of natural origin are the indolocarbazoles, such as staurosporine, and the bisindolylmaleimides. Staurosporine, a potent but nonselective protein kinase inhibitor, is a microbial alkaloid from *Streptomyces* sp. It inhibits PKA with a K_i of about 10 nM, along with many other kinases. Staurosporine is rather unselective against some kinases such as CK1 and CK2, with inhibitory kinetics in the micromolar range, a fact that has been connected to the existence of relatively bulky sidechains in the ATP pocket of these kinases (Meggio et al. 1995). Again, its widespread use in signalling research and as a lead compound for potential therapeutics was triggered by its efficient inhibitory activity

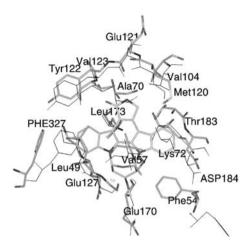


Fig. 11 Induced fit movements of enzyme residues in the vicinity of staurosporine upon binding of staurosporine to PKA (Prade et al. 1997). Residues rendered as *stick models* are from the 1STC structure, residues from 1CDK are represented as *lines*

against PKC. Staurosporine shows similar binding modes with different protein kinases. Cocrystal structures were solved from staurosporine in complex with inactive CDK2 (PDB-code 1AQ1) (Lawrie et al. 1997) and with active PKA (1STC) (Prade et al. 1997; Toledo and Lydon 1997), with CSK (1BYG) (Lamers et al. 1999), Chk1 (1NVR) (Zhao et al. 2002), MapKap kinase 2 (1NXK) (Underwood et al. 2003), Gsk3 β (1Q3D) (Bertrand et al. 2003), Lck (1QPJ) (Zhu et al. 1999), and with the AGC kinase PDK1 (Komander et al. 2003). The molecule binds to PKA with two hinge region H-bonds, one to Val123 amide and the other to the Glu121 carbonyl group. Both hydrogen bonds are conserved adenine contacts, and established in all other staurosporine structures in an identical manner. Also comparable to ATP, the staurosporine methylamino group makes contacts to both the carbonyl of Glu170 (seen also with all H-inhibitors except H-1152P) and to the sidechain of Glu127. The latter contact was also made by fasudil, but not by other H-inhibitors cocrystallized with PKA. In addition to these four hydrogen bonds, staurosporine makes a large number of VDW contacts with the enzyme, possibly because the kinase undergoes induced fit movements upon binding of the inhibitor. In order to accommodate the large staurosporine, most sidechains in the contact area of the inhibitor, the peptide backbone in the region of Val104 and Thr183, and the C-terminal stretch including residue Phe327, move by up to almost 5 Å and expand the ATP pocket (Fig. 11). Phe327, however, makes favourable edge-to-plane contacts with one of the staurosporine indoles. None of the other kinases that have been cocrystallized with staurosporine, including PDK1, possesses a residue corresponding to Phe327, which is conserved amongst most AGC kinases. Another aromatic residue that undergoes a conformational change to contact staurosporine is Phe54 at the tip of the glycine-rich loop. Phe54 bends inwards towards the inhibitor and makes favourable VDW contacts with the methyl group at the staurosporine sugar ring. A similar contact is observed in Chk1, where a tyrosine residue in the homologous position interacts with the methyl group of the staurosporine sugar. Although Cdk2, Csk, Gsk3 β and Lck have a Tyr or Phe residue at the corresponding position at the tip of the glycine-rich loop, none of them makes a similar contact with this aromat.

2.5 Structures of PDK1 with UCN01 and Staurosporine: Role of a Glutamine Switch in Ligand Binding

2.5.1 3-Phosphoinositide-Dependent Protein Kinase 1

The cellular integration of growth factor signals via membrane receptors results in the activation of the phosphatidylinositol 3-kinase (PI3 K), which in turn generates the membrane-bound second messengers phosphatidylinositol 3,4-diphosphate [PI (3,4)P2] and PI(3,4,5)P3. These second messengers recruit PKB/Akt via a mechanism that involves 3-phosphoinositide-dependent kinase 1 (PDK1), resulting in insulin regulation, cell growth and cell survival (Vanhaesebroeck and Alessi 2000). The PDK1 kinase activity was purified first from rabbit skeletal muscle (Alessi et al. 1997b) and rat brain (Stokoe et al. 1997). Human PDK1 is a polypeptide of 556 amino acids (63.1 kDa) with at least three domains (Alessi et al. 1997a): an AGC-Ser-Thr kinase domain that spans residues 84-341; a pleckstrin homology domain (PH) at the C-terminus (residues 450-550), which binds to the PtdIns(3,4,5)P3 and PtdIns(3,4)P2; and the N-domain (first 50 residues) with yet-unclear function. Phosphorylation sites in this domain for various protein kinases are assumed to play a role in PDK1 activity (Park et al. 2001; Wick et al. 2002; Kim et al. 2003; Taniyama et al. 2003).

Originally described as a PKB kinase, PDK1 can also phosphorylate and activate many Ser-Thr kinases, all of them members of the AGC subfamily. Reports to date include the phosphorylation and activation of the p70-kDa ribosomal S6 kinase (S6 K) (Alessi and Cohen 1998; Pullen et al. 1998); possibly the cAMP-dependent protein kinase (PKA) (Cheng et al. 1998); the serum- and glucocorticoid-regulated protein kinase (SGK) (Kobayashi and Cohen 1999); the p90-kDa ribosomal S6 kinase (RSK1) (Jensen et al. 1999; Richards et al. 1999) and its isoform RSK2 (Frodin et al. 2000); PKC (Le Good et al. 1998) and protein kinase C-related kinase (PRK) (Flynn et al. 2000) by PDK1.

In two-hybrid screening it was found that PDK1 interacts with a 24-amino acid fragment called PIF (PDK1-interacting fragment) (Balendran et al. 1999) corresponding to the C-terminus of PRK2. PIF is characteristic for most AGC kinases, always located downstream of the catalytic domain, and corresponds to the hydrophobic motif (HM) with the consensus sequence FXXFS/TY. Atypical PKCs and PRK isoforms have an acidic residue (Asp or Glu) instead of Ser or Thr, in the case of PKA the motif is truncated (FSEF-COOH), while PDK1 itself has no hydrophobic motif (Vanhaesebroeck and Alessi 2000). Typically, PDK1 interacts with the HM of its substrates when the HM is phosphorylated. This interaction through the phosphorylated HM is essential for the activation of RSK2 (Frodin et al. 2000), PKC (Gao et al. 2001), PRK (Balendran et al. 2000), S6 K and GSK (Biondi et al. 2001). The PIF-binding pocket of PDK1 is located in the catalytic domain, first determined by comparison with the structure of PKA and verified by the crystal structure of PDK1 (Biondi et al. 2000; Biondi et al. 2002). Further modelling studies determined that a PIF-binding pocket and a phosphate pocket are present in PDK1 and most other AGC kinases (Frodin et al. 2002).

The activation of PKB by PDK1 (through phosphorylation of the activation loop Thr308) and the presence of PI(3,4,5)P3 is rather the result of a recruitment and colocalization of both enzymes at the membrane than of the activation of either enzyme directly by the phosphatidylinositides. Consequently, the activation loop phosphorylation of the other AGC kinases by PDK1 is not enhanced by the presence of PI(3,4,5)P3. These other kinases recruit PDK1 rather via the phosphorylated hydrophobic motif. However, the regulation of the prerequisite phosphorylation of the hydrophobic motif often appears to be dependent on PI(3,4,5)P3. PDK1, in contrast to the other AGC kinases, lacks such a hydrophobic motif, but has instead a hydrophobic pocket similar to the binding site for the FXXF C-terminal hydrophobic motif of PKA (Frodin et al. 2000).

In summary, there are two models for PDK1 activation. One is for PKB, which consists of the recruitment of both kinases to the membrane by phosphatidylinositol phosphate through their respective PH domains. The proximity of both kinases facilitates the activation. The other model, valid for the rest of AGC kinases, consists of a two-step activation process. An HM kinase first phosphorylates the hydrophobic motif that is then recognized by PDK1, which in turn transfers the activating phosphate to the activation loop.

A significant number of human cancers correlate with enhanced levels of PI(3,4,5,)P3 that lead to activation of PKB and S6 K and result in enhanced cell proliferation and tumour cell survival. Thus, PDK1 as the activating upstream kinase may be a promising target for small molecule drugs that target either the hydrophobic pocket of the kinase or the ATP binding site. PDK1 has been cocrystallized with staurosporine and UCN01 (Komander et al. 2003).

UCN01 (or 7-hydroxystaurosporine) is a derivate of staurosporine with an additional hydroxyl group on the lactam ring. UCN01 was isolated, as staurosporine, from *Streptomyces* sp. and was originally believed to be selective for PKC, but in fact inhibits several other kinases (Davies et al. 2000; Gescher 2000). UCN01 is undergoing clinical trials because of its property to inhibit growth and induce apoptosis of cancer cells. Staurosporine and UCN01 bind to PDK1 very much as staurosporine binds to PKA. Both form the corresponding hydrogen contacts from the lactam ring to the hinge backbone, and both make contacts from the methylamino group to Glu166 (Glu127 in PKA) and to the backbone carbonyl of Glu209 (Glu170 in PKA). In contrast to PKA, the Phe93 (Phe54 in PKA) does not bend inwards to contact the staurosporine hydroxyl group. The reason may be again the exchange of the Gly55 homologue to serine (Ser94) in PDK1.

2.5.2

PDK1 Cocrystals with Staurosporine and UCN01

The inhibitory profile of UCN01 across a panel of several protein kinases is different from that of staurosporine. Since the binding modes of both inhibitors is identical in PDK1 and in Chk1 (PDB-code 1NVQ) (Zhao et al. 2002), selectivity must be defined by the environment of the 7-hydroxy group of UCN01. This group makes a direct hydrogen bond to Thr222 (Thr183 in PKA), usually reserved for a contact of the ATP N7. The kinases that are inhibited most strongly by UCN01 have a corresponding Thr or Ser residue, either in this position or in the position of Val 143 (Val104 in PKA), which could make a similar contact to the 7-hydroxy group. It is assumed that kinases which are inhibited more potently by staurosporine than by UCN01 appear to have bulkier sidechains in the environment of the 7-hydroxy group (Johnson et al. 2002b), such as Met120 in PKA or Phe80 in CDK2.

In addition, the 7-hydroxy group binds via water to the sidechain of a glutamine residue (Gln220), which changes its conformation towards the ATP pocket in order to establish this interaction. This rotamer change of this glutamine residue in association with the occupation of the binding site is especially noteworthy because it was previously observed in a mutant of PKA (Gaßel et al. 2003). In the attempt to design a surrogate kinase for PKB based on PKA, Val123 (the hinge region residue that is involved in almost all inhibitor, enzyme backbone contacts) was mutated to alanine, and Leu173 to methionine. Unexpectedly, the mutant showed defects (tenfold reduction in affinity) in binding of ATP and inhibitors not explained by these two conservative sidechain exchanges. In a crystal structure of the mutant apoenzyme, the reason for the kinetic defects became clear: Gln181, located close to the hinge region but in the wild-type enzyme, is directed outwards towards the surface of the enzyme and adopts a different rotamer and occupies the space freed by the exchange of Val123 to alanine, partly obstructing the adenosine pocket. In a cocrystal structure with MgATP, the residue

adopts its normal conformation again. The conformational change, however, apparently costs energy, as ligand binding is weakened. An additional mutation of this Gln181 to lysine, conserved in most other kinases, restores the basic situation, with the lysine adopting the orientation of Gln181 in wildtype PKA or the corresponding lysine in Chk1 or PKB. Interestingly, PDK1 is the only kinase which has a glutamine residue in the 181 position as PKA, and an alanine sidechain in the Val123 position as PKB, which might lead to the expectation of a possible similar conformational change of the glutamine sidechain towards the adenosine pocket as observed in the PKA-to-PKB mutant. As the structure with UCN01 confirms, this is indeed the case. Although an in vivo relevance of this situation is not clear, such a behaviour in principle offers a way to communicate the occupation of the ATP binding site to the surface of the enzyme (Gaßel et al. 2003) and represents an unexpected mechanism for inhibitor selectivity. In a possible analogy, a glutamine residue in the allosteric site of glycogen phosphorylase is known to change its conformation upon phosphorylation of the enzyme and to hinder access of glucose-6-phosphate (Sprang et al. 1988).

2.6

PKA Cocrystal Structures with Bisindolylmaleimide Inhibitors Selective for PKC

2.6.1 Protein Kinase C

Discovered in 1977 (Inoue et al. 1977; Kishimoto et al. 1977), PKC is one of the most intensely studied subjects in signalling research. In humans, 10 PKC isoforms are present, confirmed by the analysis of the human genome (Manning et al. 2002). Besides the catalytic core domain, which is conserved among PKA, PKB and PKC with more than 40% identity, the PKC isoforms have additional domains, which allow the interaction with a number of activity-modulating substances.

Three distinct subfamilies of PKC isoforms can be defined according to their essential activators: (1) conventional PKCs (α , β I/II, γ), which require phosphatidylserine (PS), diacylglycerol (DAG) and Ca²⁺; (2) novel PKCs (δ , ϵ , η , θ), which need PS and DAG but they are insensitive towards Ca²⁺; and (3) atypical PKCs (ζ , ι), which are insensitive to both DAG and Ca²⁺, although PS regulates activity (for reviews see Way et al. 2000; Newton 2003 and citations therein). Furthermore, additional lipid mediators, like fatty acids and lysophospholipids, have been shown to influence the catalytic activity of PKCs (Nishizuka 1995). Interaction of PKCs with the activators leads to phosphorylation of a threonine residue on the activation loop of all PKC isoforms and additionally of a serine or threonine residue in the hydrophobic motif of the conventional and novel PKCs. The atypical PKCs possess a glutamate at the hydrophobic motif phosphorylation position that performs the activation function (for reviews see Newton 2001, 2003). PKCs are involved in the signal transduction cascades that regulate proliferation, apoptosis, contraction, migration, hypertrophy, secretion and further growth factor-dependent processes (for review see Dempsey et al. 2000). PKC α , for example, is involved in regulation of proliferation, apoptosis, differentiation, cell migration and adhesion, among other cellular and pathogenic processes (for a review see Nakashima 2002). Consistent with its prominent role in different signal transduction pathways, PKC is implicated in different diseases. The earliest studies associated PKC with cancer via the influence of the tumourpromoting phorbol esters (Nishizuka 1984; Castagna et al. 1982). The effects of PKCs in the field of cancer are manifold (for review see Hofmann 2001); for example, whereas PKC α , PKC β II, PKC ϵ and the atypical PKC isoforms appear to be antiapoptotic, the PKC δ isoform is activated by caspase-induced proteolysis and may serve to amplify downstream processes in the apoptotic pathway (for review see Dempsey et al. 2000). Many molecules with PKC-modulatory activity have been developed to elucidate PKC signalling in cancer and other diseases. Early compounds have often been naturally occurring substances (e.g. staurosporine from Streptomyces sp.), whereas the more recent ones have been developed with an increased selectivity. Three classes of protein kinase C modulators have been developed so far, grouped by their mode of action:

- a. ATP-competitive inhibitors which bind in the catalytic site of protein kinases (e.g. indolocarbazoles, bisindolylmaleimides, balanols, flavonoids and phenylaminopyrimidines)
- b. Inhibitors that that bind to one of the regulatory domains of an PKC isoform (e. g. phospholipid and sphingolipid analogues, lactones and antiestrogens)
- c. An antisense oligonucleotide that binds to the mRNA (for review see Tamm et al. 2001)

The antisense oligonucleotide ISIS3521 binds isoenzyme specific to the messenger RNA of PKC α and shows antitumour activity in human glioblastoma, breast and lung xenograft models (Tamm et al. 2001; Swannie and Kaye 2002). The most important inhibitor with the ability to bind to regulatory domains of a PKC isoform is bryostatin, which belongs to the group of macrocyclic lactones derived from the marine bryozoan *Bugula neritina* (Pettit et al. 1970). The binding of bryostatin leads after an initial activation to a down-regulation of PKC activity (for review see Hofmann 2001) and is in clinical trials against various cancers (e.g. melanoma, non-Hodgkin's lymphoma, colorectal, renal) both as a single agent and in combination with chemotherapy (for review see Swannie and Kaye 2002).

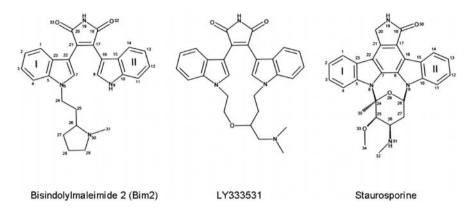


Fig. 12 Cognates of the bisindolylmaleimide/indolocarbozole group of protein kinase inhibitors. Bisindolylmaleimide 2 (BIM2) has been cocrystallized with a PKA mutant and with PDK1 (Gaßel et al. 2004; Komander et al. 2004); LY333531 (ruboxistaurin) has been cocrystallized with PDK1 (Komander et al. 2004); staurosporine has been cocrystallized with several kinases including PKA [(Prade et al. 1997) PDB code 1STC]

Besides these interesting compounds, a number of ATP-competitive inhibitors have been shown to down-regulate PKC activity. The earliest members of this group are the indolocarbazole staurosporine, which binds with high affinity to PKC, and the flavonoid quercetin with a low-affinity binding mode. Both exhibit a broad spectrum of interacting protein kinases and other enzymes. The staurosporine derivative PKC412 (N-benzoyl-staurosporine, CGP41251) originally designed as a PKC inhibiter, is indeed more specific than staurosporine but has significant activity against various other kinases, including KDR (kinase insert domain containing receptor), vascular endothelial growth factor (VEGF)-R2, platelet-derived growth factor (PDGF) and c-Kit. PKC412 shows in vitro and in vivo antiproliferative activity accompanied by a cell-cycle arrest at the G2/M state and polyploidy (Fabbro et al. 2000). Furthermore, PKC412 modulates the P-glycoprotein-mediated drug resistance (Ganeshaguru et al. 2002) via PKC inhibition and shows antiangiogenic action, which is related to its activity towards VEGF (Fabbro et al. 2002). It is in clinical trials for treatment of neovascular retinopathy and different kind of cancers. Although broad unspecificity is probably a pharmacological disadvantage, some crystal structure complexes with these compounds have been solved. However, no PKC crystal structure has been reported to date, despite the fact that PKC has been known as an interesting pharmacological target for years. Available cocrystal structures of PKC inhibitors with a related kinase are those of staurosporine and its closely related derivative UCN01 (see above) and the bisindolylmaleimides (see below) (Fig. 12). Besides cocrystallization with PKA (PDB code 1STC) (Prade et al. 1997), the extended and rigid planar staurosporine has been crystallized

with Cdk2 (PDB code 1AQ1) (Lawrie et al. 1997) Src kinase (PDB code 1BYG) (Lamers et al. 1999) and others. UCN01 (7-hydroxystaurosporine) has been cocrystallized with Cdk2 (PDB code 1PKD) (Johnson et al. 2002b), Chk1 (PDB code 1NVQ) (Zhao et al. 2002), and PDK1 (Komander et al. 2003). Furthermore, quercetin, which shows a weak inhibitory effect on PKC, has been cocrystallized with the Src kinase Hck (PDB-code 2HCK) (Sicheri et al. 1997).

2.6.2 Cocrystal Structures of PKA with BisindolyImaleimides

Recently, we were able to solve the structure of a flexible derivative of staurosporine, namely bisindolylmaleimide 2 (BIM2) in complex with PKA (PDB-code 1SZM) (Gaßel et al. 2004) (Fig. 12). The bisindolylmaleimide class of PKC inhibitors is derived from staurosporine by elimination of a single bond. This converts the extended planar aromatic group into the three aromats described in the compound name, with corresponding additional degrees of freedom. The resulting dimer shows unusual conformations of the kinase as well as of the inhibitor. The kinase molecule A of the dimer is in general comparable to the kinase structure of 1STC (Prade et al. 1997), with two major differences: (1) the whole turn region including residues Gly52, Ser53, Phe54 and Gly55 of the glycine loop (and not merely the sidechain of Phe54) is turned down, probably to enable a contact between the methyl group of the pyrrolidine-tail of BIM2 and phe54 of the glycine loop; (2) the linker region around Phe327 is flexible (not resolved in the electron density) in the BIM2 structure, possibly due to lesser interaction with the inhibitor compared to staurosporine and all other inhibitors crystallized with PKA so far (Fig. 13). In contrast to the planar and rigid molecule staurosporine, the twisted conformation of the indole rings limits the number of possible VDW contacts to residues Val57 and Leu49 from the glycine-rich loop. While the methylamino group of staurosporine mimics the interaction of the ATP-ribose hydroxyl groups with the backbone carbonyl of Glu170 and the sidechain of Glu127, the pyrrolidine moiety of BIM2 does not establish these interactions. In the presence of BIM2, both residues adopt different rotamers: Glu127 to avoid a steric conflict with the pyrrolidine group, which prevents any H-bond contact with the inhibitors, and Glu170 to establish a sidechain contact to the amino group the pyrrolidine ring. Both residues are key residues for recognition of the substrate consensus sequence of PKA and probably other AGC kinases such as PKC also.

The molecule B of the dimer is in the most open conformation so far described for PKA. In addition to the common opening hinge movement around amino acid residue 125 (Olah et al. 1993) of PKA structures in open conformations such as 1STC (Prade et al. 1997), or the apoenzyme structures (1CTP) (Karlsson et al. 1993) and 1J3H (Akamine et al. 2003), the ki-

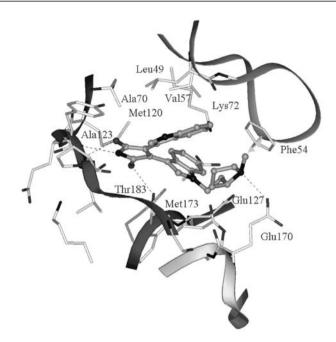


Fig. 13 Binding pattern of BIM2 in PKA molecule A (intermediate open conformation of the enzyme). Inhibitor-interacting residues are indicated. The hinge region H-bonds Ala123:N-O32 and Glu121:O-N19 are supplemented by Thr183OG1—O33 (Gaßel et al. 2004). Conserved parts of the backbone are indicated as *ribbon*: the hinge region, the metal binding loop, and the catalytic loop

nase in molecule B shows a rotation or twist of its N- and C-lobe by 15 degrees when compared with molecule A of 1J3H. This rotation is clockwise for the N-lobe when looking from above the N-lobe towards the C-lobe. The axis of this rotation goes through the centre of both N- and C-lobes. Furthermore, the hinge region is displaced by up to 5 Å out of this common position. These considerable movements of the kinase domains in molecule B are induced by the reversed binding of BIM2, which is possible because of the symmetrical nature of the maleimide head group (Fig. 14). The binding of BIM2 in molecule A and molecule B is similar with respect to the hinge contacts of the maleimide groups, and the VDW contacts to Ala70, a conserved residue from β -strand 3. An H-bond from the maleimide carbonyl to the sidechain of Thr183 is established only in molecule A. Although the bisindolylmaleimide scaffold itself is apparently symmetric, the pyrrolidine substitution on indole I breaks the symmetry and defines an overall orientation. Thus, while the hydrogen bonding pattern of the maleimide and hinge loop remains the same between the two binding modes, the hinge hydrogen bond acceptor of the inhibitor is alternately the oxygen distal or proximal to the substituted indole I in molecules A and B, respectively. This upsidedown

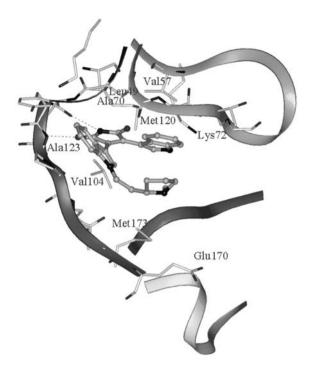


Fig. 14 Binding pattern of BIM2 in PKA molecule B (open conformation). Inhibitor interacting residues are indicated. The C-terminal lobe residues Glu127, Glu170 and Met173 are not in contact with the inhibitor. Hinge region H-bonds exist between Ala123:N–O33 and Glu121:O–N19 (Gaßel et al. 2004). Different parts of the backbone are indicated as *ribbons*: the hinge region, the metal binding loop, and the catalytic loop

orientation of the maleimide affects the relative orientations of the indole moieties by the means of the different interacting residues of the kinase. An exception is Val57, which is, in both orientations of the inhibitor, one of the coordinating residues of the inwards-rotated indole II. The orientation of the pyrrolidine is in both cases parallel, in a VDW-contact distance to the indole I. The pyrrolidine of BIM2 in molecule B seems to be coordinated only by this intramolecular interaction, because no contacts to the kinase are established. As a result, BIM2 adopts two very different conformations, with different rotamers for the pyrrolidine-tethered indole I (delta chi 90°) and the untethered indole II (delta chi 30°). In PKA, BIM2 is bound to the same kinase under identical conditions in two different inhibitor conformations to two different conformations of the protein, possible because of the symmetric nature of the maleimide head group. This apparent directing effect of the maleimide group on the overall binding pattern might be a useful feature to explore in future inhibitor design.

2.6.3 Structures of PDK1 with BisindolyImaleimides and LY333531

Bisindolylmaleimides were also cocrystallized with PDK1 (PDB-codes 1UU7, 1UU8, 1UU9, 1UVR) (Komander et al. 2004). The structure obtained with PDK1 and BIM2 is very similar to the molecule A of our structure of PKA and BIM2, but an alternative conformation comparable to the molecule B structure of PKA with BIM2 was not observed in the case of PDK1. The hinge region contacts of the bisindolylmaleimides are conserved between the two kinases. As in PKA, BIM2 in PDK1 also shows the inwards rotation of the untethered indole. As one of the rare AGC kinase without a comparable residue to Phe327, the PDK1 structure also lacks these contacts between the inhibitor and the kinase, in this respect similar to the structure of PKA, where the corresponding peptide stretch is mobile and not resolved. As a striking difference between the two BIM2 kinase complexes, the glycine-rich loop in PDK1 shows no conformational change similar to that in PKA, and no contact between the Phe54 homologue in PDK1 is made to the pyrrolidine-attached methyl group of BIM2. A possible reason is the exchange of Gly52 (PKA) to serine in PDK1, which reduces the flexibility of the glycine flap and might cause steric problems. As in the less-pronounced case of the Phe54 interaction with the staurosporine sugar methyl group in PKA, where other kinases that possess the corresponding residue and still do not make the contact, the real reasons for the presence or absence of this interaction may be different.

The presence of a PKA molecule which contains an upsidedown-oriented BIM2 inhibitor, in contrast to PDK1, may be facilitated by Pro124 in PKA. In the B molecule in PKA, BIM2 is wedged between Val57 from the N-lobe, and Pro124, which makes one-sixth of all VDW contacts to the inhibitor.

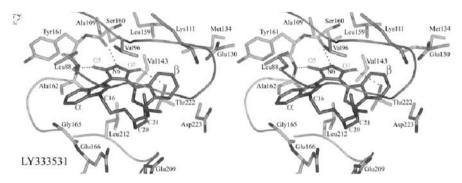


Fig. 15 Stereo representation of LY333531 interactions within the ATP binding pocket of PDK1. Ligand carbon atoms are in *dark grey* and protein carbon atoms in *light grey*. Hydrogen bonds are indicated by *black dotted lines*. A *grey ribbon* indicates the protein backbone. (Komander et al. 2004)

The corresponding residue is a lysine in PDK1, which would not be suitable for similar interactions.

Another PKC inhibitor of the bisindolylmaleimide class, LY333531 or ruboxistaurin, has also been cocrystallized in complex with PDK1 (PDBcode 1UU3) (Komander et al. 2004) (Fig. 15). LY333531 shows PKC isoform specificity (e.g. 80- and 60-fold selectivity for PKC β I and PKC β II over PKC α (Jirousek et al. 1996) and is in phase III clinical trials for diabetic retinopathy and diabetic macular oedema (Frank 2002 and citations therein). LY333531 shares much of the flexibility of BIM2, except that the cyclic connection of both N-atoms of the indoles with a 6-atom ether linker prevents a comparable inwards rotation of one indole as seen with bisindolylmaleimide 2 (Fig. 12). Komander et al. explain the higher binding affinity of inhibitors such as staurosporine, UCN01 and LY333531 in contrast to bisindolylmaleimides by a preference of PDK1 for carbon atoms at specific positions which are satisfied by the indolocarbazoles and LY333531 but not by the bisindolylmaleimides. Mutagenesis of PDK1 and sequence comparisons, however, did not explain the selectivity of the bisindolylmaleimides and LY333531 for the PKC isoforms.

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Inhibitors of Protein Kinase CK2: Structural Aspects

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Abstract Protein kinase CK2 is one of the most challenging members of the kinase superfamily. Although this protein has been the subject of intensive studies over the last 50 years, very little is known about its precise biological function and mode of regulation. The CK2 holoenzyme is composed of two catalytic α - and two regulatory β -subunits and is classified as an acidophilic Ser/Thr kinase. Unique properties of the catalytic α -subunit are its intrinsic activity and high pleiotropicity. CK2 is supposed to be involved in many fundamental aspects of the normal cell life as well as in degenerative processes that can lead to cancer or tumor pathologies. This makes CK2 an interesting target for the development of inhibitors with pharmacological perspectives. The inhibitors studied are directed to the CK2 ATP-binding site that, among the known kinases, carries some distinctive features as indicated by its ability to use both ATP and GTP as co-substrates and the low susceptiveness to staurosporine inhibition. On the basis of three-dimensional crystal structures, we describe and discuss the effects of the binding to CK2 of inhibitors with a potency in the low micromolar range belonging to different chemical families, i.e., ben-

zotriazoles, anthraquinones, and quinazolinones. The overall structure of the protein is poorly affected by the binding of these small molecules. In the proximity of the binding site, the most affected residues are Asn118, His160, Met163, and those of the glycine-rich loop. Two of the inhibitors, namely tetrabromo-2-benzotriazolo (TBB) and the indoloquinazolinone IQA, display a significant selectivity among panels of tens of different kinases. An important common energetic contribution to the inhibitors' binding is ascribed to the hydrophobic interaction with the apolar surface region of the CK2 binding cleft. The shape and the reduced dimension of the CK2 active site in comparison with other kinases are essential in explaining the selectivity of these inhibitors as well as the anomalous low potency of staurosporine.

Keywords CK2 · Kinase inhibitors · Complexes · Crystal structure · Anthraquinones · Benzotriazoles · Quinazolinones

1 Protein Kinase CK2

One of the first members of the protein kinase superfamily ever discovered is CK2, formerly known as "casein kinase 2." The first report, dated 1954 by Burnett and Kennedy, described this enzyme isolated from liver extracts along with protein kinase CK1 (Burnett and Kennedy 1954). For their experiments, the authors utilized casein as an artificial substrate, and this is the reason for the early name casein kinase 2 (indeed, since casein is not a natural substrate for the enzyme, and since the real ones are still undefined, the name was moved to the more generic "CK2").

Protein kinase CK2 is an eukaryotic acidophilic Ser/Thr protein kinase. This ubiquitous enzyme is well conserved throughout evolution and, based on a recent classification of the human kinome (Manning et al. 2002b; see also the web-site at http://kinase.com/kinbase/), the catalytic subunit belongs to the so-called other kinase group, i.e., without strong similarities to other groups, giving origin to the CK2 family. Among the nearest relatives are the cyclin-dependent kinases, belonging to the CMGC group.

The holoenzyme comprises a tetrameric assembly formed by two catalytic (α) and two regulatory (β) subunits. Depending on the species, several catalytic and regulatory paralogs have been identified; in humans, two catalytic (α - and α') subunits and one regulatory (β) subunit are known.

Owing to some peculiar properties, CK2 is considered a quite anomalous protein kinase (Pinna 2002): (a) it is highly pleiotropic, (b) it can use ATP and guanosine triphosphate (GTP) as co-substrates, (c) the target serine or threonine must be surrounded by acidic residues and (d) the α -catalytic subunit is intrinsically active. Finally, most importantly, the precise function of this enzyme has not been deciphered. For recent reviews on the subject, see references (Pinna 2002; Litchfield 2003; Meggio and Pinna 2003).

2 Substrate Pleiotropicity and Function

To date, more than 300 substrates are known for CK2 (Meggio and Pinna 2003). Although most of these substrates are phosphorylated on serine or threonine residues, and consequently CK2 is classified as a Ser/Thr kinase, some examples of tyrosine phosphorylation are also documented (Chardot et al. 1995; Marin et al. 1999). In this respect, evidence was presented that indeed both the α - and α' -catalytic subunits of CK2 undergo an intermolecular autocatalytic event on a tyrosine placed in the activation loop, very likely Tyr182 (Donella-Deana et al. 2001). The high pleiotropicity is a distinctive feature of this kinase. Looking at the classes of proteins that are phosphorylated, it has been assumed that CK2 is involved in many aspects of cell cycle regulation, cell growth and differentiation, embryogenesis, and, recently, apoptosis. Abnormally high levels of the enzyme detected in transgenic mouse lymphomas (Seldin and Leder 1995) and in solid tumors (Guerra and Issinger 1999) have suggested the involvement of CK2 in tumorigenesis phenomena (Tawfic et al. 2001). Nevertheless, while both CK2 catalytic and regulatory subunits have been demonstrated essential for cell viability in Saccharomyces cerevisiae (Padmanabha et al. 1990), in Caenorhabditis elegans (Fraser et al. 2000), and in mice (Buchou et al. 2003), the precise functions of this enzyme are not well understood. Recently, it has been proposed that CK2 also has a crucial role in the transduction of survival signals, protecting the cell against stress events (Ahmed et al. 2002).

3 Constitutive Activity and Lack of Regulatory Mechanisms

Mechanisms regulating the catalytic activity of most protein kinases that have been recognized to date include the control by additional subunits or domains and phosphorylation and dephosphorylation events (Johnson et al. 1996; Huse and Kuriyan 2002). In the regulation of most, but not all, protein kinases, also a (auto)phosphorylation process takes place in the activation loop, often at a residue corresponding to the threonine-197 in cAMP-dependent protein kinase (cAPK) [for instance in cdc2 and mitogen-activated protein kinase (MAPK)]. Despite CK2's discovery almost 50 years ago and the numerous studies that followed, the regulatory mechanism is still the subject of intense discussion, and none of the aforementioned regulations looks to be appropriate.

The catalytic subunit of this enzyme is intrinsically active, even in absence of the regulatory segment; once the holoenzyme is formed, its activity can increase or decrease, depending on the substrate involved. It was suggested (Pinna 2002) that, unlike many other kinases that are usually inactive in the cell and are activated only transiently, CK2 is regulated in the opposite manner; i.e., when present, it is normally active and is regulated by inactivation. To make the scenario even more complicate, recently it has been proved that the dimeric β -subunit can indeed interact with several partners other than the CK2 α -subunit, suggesting for the β -subunit a more complicated role (Litchfield 2003). Evidence of possible distinctive roles for the isolated catalytic and regulatory subunits has led to the hypothesis that a balance in the expression of the two subunits together with their different specific localization in the cell compartments can contribute to the regulation of CK2 functions (Faust and Montenarh 2000).

The constitutive activity of CK2 has also been postulated to be responsible for the exploitation of this kinase by an increasing number of viral agents for the phosphorylation of proteins essential for their cell cycle.

4 General Structural Features of Ser/Thr Protein Kinases Catalytic Domain

Ser/Thr protein kinase catalytic domains are structurally well conserved throughout evolution, from prokaryotes to mammals, while their amino acid sequences show a much lower degree of similarity (Manning et al. 2002a; Young et al. 2003). Nevertheless, residues essential for the function of the enzyme are conserved in all kinases that invariably share some characteristic properties (Hanks and Hunter 1995). Figure 1 shows an alignment between the sequences of cAPK, that is often considered as the reference prototype kinase, and the CK2 catalytic α -subunit from human and *Zea mays*, with the most important residues highlighted.

The overall three-dimensional structure comprises both β -strands and α helices, with a large predominance of the former in the N-terminal lobe and of the latter in the C-terminal one (Fig. 2).

Between the two lobes an essentially hydrophobic deep cleft is formed, where the co-substrate (ATP or, less frequently, GTP) can bind. The two lobes are connected by a segment known as the hinge region, where the adenine moiety of ATP can be anchored by means of some hydrogen bonds.

In order to be active, the conformation of a kinase must guarantee the access of cosubstrate and substrate to the catalytic zone and the correct arrangement of the catalytic and binding residues; in particular, the proper orientation of ATP is crucial for catalysis. The binding of the ATP phosphate tail to the enzyme is favored by two metal ions, usually magnesium, that are coordinated by an asparagine residue of the so-called catalytic loop (Asn161 in CK2, 171 in cAPK) and an aspartate at the beginning of the activation segment (Asp175 in CK2, 184 in cAPK).

Two other structurally conserved important zones in kinases are the Glyrich loop, or p-loop, and the helix α C (Fig. 2); the Gly-rich loop contributes

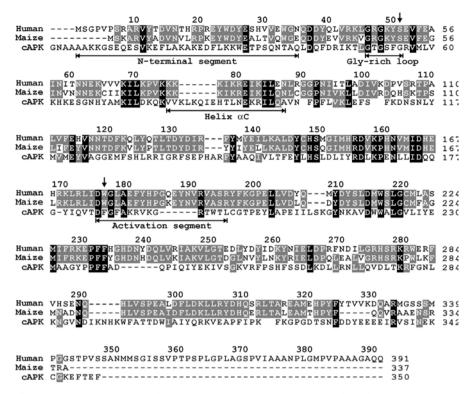


Fig. 1 Sequence alignment of cAPK and CK2 catalytic α -subunits from human and maize. *Vertical arrows* mark important residues not conserved between cAPK and CK2, as discussed in the text. The structurally and functionally important regions of CK2 are indicated. *Numbering in the upper line* refers to human CK2 sequence. *Black background*, residues identical in all three sequences; *gray background*, residues identical in only two sequences

to strengthen the ATP anchoring, while the orientation of the α C helix is essential for the activity of the enzyme. In Ser/Thr kinases that present inactive and active forms, as in the case of cAPK or CDK2, helix α C has been found with an orientation substantially different in the two arrangements (Jeffrey et al. 1995). So, when a transition from an inactive to an active structure occurs, there is usually a rearrangement of the relative orientation of the two lobes, to properly position essential residues of the catalytic site (for instance those corresponding to Lys72 and Asp166 in CDK2) and a concomitant correct positioning of the α C helix in the N-terminal domain. Another important structural change occurring for the activation is represented by the displacement of the activation segment, a long loop in the C-terminal domain, that moves away from the close position of the inactive form to an open one (Fig. 2), allowing the binding of the substrate and co-substrate to

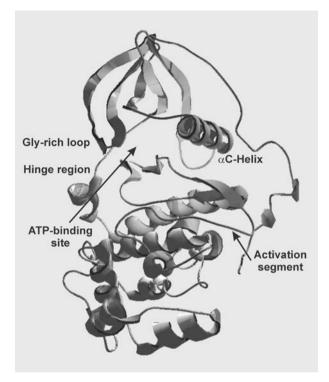


Fig. 2 Three-dimensional structure of Apo-CK2. Regions important in all Ser/Thr kinases are indicated. The N-terminal region (*upper part*) is rich in β -sheets while the C-terminal one (*lower part*) is rich in α -helical structure. ATP binds in the cleft between the two lobes, adjacent to the Gly-rich loop and the hinge region. The activation segment adopts an open active conformation

the enzyme (Johnson et al. 1996). This movement can be caused by a phosphorylation event, as in the case of cAPK when Thr197 is phosphorylated, sometimes coupled with an interaction with e regulatory subunit, as in the case of CDK2 with cyclin. The activation segment displacement is made possible principally by a rotation of a short stretch of three amino acids positioned at the beginning of the loop; this tripeptide, Asp-Phe-Gly, is highly conserved in kinases but not in CK2, where the phenylalanine is substituted by a tryptophan at position 176 (indicated by a vertical arrow in Fig. 1). The C-terminal end of the activation segment, the p+1 loop, is responsible for substrate recognition and binding.

It must be noted, however, that not all kinases show a dramatic transition from an active to an inactive form as exhibited by CDK2 and cAPK. As already mentioned, CK2 is one of the few examples being found solely in the active form.

5 Three-Dimensional Structure of CK2 Catalytic lpha-Subunit

The first three-dimensional structure of a catalytic subunit of CK2 is that from *Zea mays*, appeared in 1998 (Niefind et al. 1998). Due to the high degree of identity with the human enzyme, CK2 from maize has been considered a suitable model for all the subsequent inhibition studies. This hypothesis has been validated by the recent determination of the crystal structure of the human holoenzyme (Niefind et al. 2001) and by those of the human catalytic subunit and a mutant independently determined by two different groups (Ermakova et al. 2003; Pechkcova et al. 2003). In all these structures the α -subunit shows essentially the same features of that from maize, particularly as far as the active site is concerned.

The CK2 catalytic subunit bears most of the sequence and structural features common to all Ser/Thr kinase. Regarding the primary structure, two notable exceptions are: (a) in the Gly-rich loop (residues 46–51 in CK2) the third glycine (indicated by a vertical arrow in Fig. 1) in the consensus sequence GXGXøG is missing (ø is usually a tyrosine, as in CK2, or a phenylalanine); (b) at the beginning of the activation segment a tryptophan (residue 176 in CK2) substitutes a phenylalanine in the otherwise conserved threepeptide Asp-Phe-Gly (Fig. 1).

Another distinctive feature of CK2 is the presence of a basic cluster at the beginning of helix α C (residues 74–80), where 6 out of 7 consecutive amino acids are basic (5 lysines and one arginine). The presence of this basic cluster, also known as a substrate recognition site, is supposed to be in relation with the attitude of CK2 to phosphorylate highly acidic substrates with the minimal consensus sequence Ser/Thr-X-X-Asp/Glu (Meggio et al. 1994). Other basic amino acids present in the p+1 loop at the C-terminal end of the activation segment, namely Arg191, Arg195 and Lys198, have been found relevant for substrate recognition (Sarno et al. 1996).

The elucidation of the three-dimensional structure of the catalytic domain of CK2 has unveiled the origin of the intrinsic activity of the enzyme (Niefind et al. 1998), showing that CK2 bears all the same structural properties responsible for the active state of cAPK, taken as the prototype of an active Ser/Thr kinase (Engh and Bossemeyer 2002). In CK2 many of the N-terminal thirty residues make several hydrophobic and polar interactions with some other important regions of the protein, located both in the N-terminal and C-terminal domains, contributing to the stabilization of the active conformation. Of particular interest are the interactions with the activation segment, which consequently is constrained in an open active state (Fig. 2). These interactions are unique among the protein kinases whose structure is known, and indeed they occur between residues highly conserved in CK2 s from different species but not among other kinases. The presence of the tryptophan-176 instead of a phenylalanine, distinctive of CK2, contributes to block the activation segment in the open active conformation; that because a hydrogen bond between the nitrogen of the tryptophan indole ring and the backbone carbonyl of Leu173 hampers the possibility of the rotation necessary for the transition from an inactive to an active conformation, possible if a phenylalanine is present instead.

The active conformation of the enzyme is granted also by the exact relative orientation of the N- and C-terminal lobes, by the proper position of the substrate and co-substrate anchoring and catalytic residues of the active site and by the correct orientation of the α C helix, spanning residues from 74 to 89. In particular, Glu81 contributes, through an ionic interaction, to orient Lys68 in the optimal position for the proper alignment of the ATP phosphates for catalysis. The importance of Lys68 and Glu81 (numbering is referred to CK2 sequence) is underlined by their conservation in all kinase sequences known to date.

In the hinge region of CK2, three are the residues involved in the nucleotides binding: Glu114, Val116 and Asn118. In the case of ATP, two hydrogen bonds between N1 and N6 of adenosine and backbone carbonyl of Glu114 and amide of Val116 are present; these interactions are typical of many kinases. In the case of GTP, atoms N1 and O6 of the purine moiety are hydrogen bound to the amide and the carbonyl of the backbone of Val116. Asparagine 118 is involved in the coordination of both co-substrates, ATP and GTP, also with the involvement of a water molecule mediating the interaction with the co-substrates. The dual co-substrate specificity is achieved by means of two well-structured water molecules that allow the switching between the different coordination modes of ATP and GTP through an hydrogen bond frame shift (Niefind et al. 1999).

6 CK2 Tetrameric Assembly

In vivo, human CK2 is found mainly, although not exclusively, in a tetrameric assembly, composed of two catalytic α - (or α') and two regulatory β -subunits. Evidence has accumulating that these subunits can exist in vivo also in isolated forms, with specific functions (Pinna and Meggio 1997). While the catalytic subunit, when isolated, is found in a monomeric state, the regulatory subunits have a strong tendency to form dimers, due to the presence of a zinc-finger motif (Chantalat et al. 1999). This dimeric state is spontaneously formed in solution and is thought to be responsible for the recruitment of the two catalytic subunits in order to assemble the final tetramer (Graham and Litchfield 2000).

The three-dimensional structure of the human holoenzyme, carrying α -subunits lacking of the C-terminal tail, was determined at 3.1 Å resolution

(Niefind et al. 2001). Even if the quality of the final model at this resolution is limited, nevertheless it clearly indicates that the catalytic subunit is poorly affected by the presence of the regulatory one. In other words, the inclusion in the tetramer does not significantly modify the structure of the isolated human catalytic subunit. The overall structure of the holoenzyme shows a "butterfly" shape, where the regulatory dimer, whose structure is very similar to that in the isolated form, binds two α -subunits that do not interact with each other. The surfaces of interaction between the subunits are not extensive, and this is somewhat unexpected due to the remarkable resistance to the action of denaturing agents showed by the holoenzyme. The interpretation of the large amount of biochemical and functional data accumulated in the years is not always straightforward in terms of the structural properties unveiled by the three-dimensional structure of the holoenzyme. This analysis is currently underway and is subject of debate in the field.

7 Protein Kinase Inhibitors

Interest in the development of protein kinase inhibitors has grown so enormously in the last decade, both in academic and industrial laboratories, that now the protein kinase family is the second most important drug target (Cohen 2002). This is mainly due to the involvement of the superfamily of protein kinases in many key functions of cell life, including cell cycle regulation, development, proliferation, signal transmission, and apoptosis. Abnormalities in the natural roles of this class of enzyme are often associated with human diseases, especially cancer and tumor pathologies, whose treatment has so far been restricted to cytotoxic and hormonal agents (Goel et al. 2002). Even though the application of kinase inhibitors as drugs is a very challenging task, mostly because of the conservation of structural features within the ATP-binding clefts, now many kinase inhibitors are in clinical trials or even in the market, the majority of them as anti-tumor drugs (Cohen 2002).

The estimate that at least 500 different protein kinases are encoded in the human genome makes really challenging the design of molecules that specifically target a single protein without affecting closely related kinases. The targeting of the ATP-binding site has the obvious drawbacks of the existence of other ATP-utilizing proteins and the high intracellular concentrations of ATP. Although peptide inhibitors directed to the substrate binding site may in principle offer a higher degree of specificity, they have their own set of problems that includes low affinity and potential bioavailability issues.

One of the first kinase inhibitors discovered has been staurosporine, that shows a large activity spectrum and therefore a low selectivity. Most protein kinases are inhibited by staurosporine with IC_{50} in the low nanomolar

range. Interestingly, CK2, as well as a few other kinases, is less affected by this inhibitor, with IC_{50} in the micromolar range. This trait, together with the quite unique ability of CK2 to utilize both ATP and GTP as phosphate donor, suggests an appreciable difference in the structural properties of the active site that potentially can be exploited in the inhibitors optimization process.

The most common chemical scaffolds used as ATP site-directed kinase inhibitors are derivatives of heterocyclic compounds, such as quinazolines, phenylamino- or pyrido- or pyrrolo- or pyrimido- or pyrazolo-pyrimidines, pyrrolo-pyridines, indolin-2-ones, purines, pyridinyl- or pyrimidinyl-imidazoles, and phthalazines. Some other building blocks are derived from natural products, such as alkaloids, flavonoids, and the aforementioned staurosporine (Garcia-Echeverria et al. 2000).

Recently, an insulin receptor tyrosine kinase bisubstrate inhibitor competitive against both nucleotide and peptide substrates has been designed (Parang et al. 2001). The crystal structure of its complex with the protein tyrosine kinase (PTK) has confirmed the double anchoring scheme of target binding. This bisubstrate inhibitor could be a precursor of a new interesting class of anticancer therapeutic agents, allowing the possibility to take advantage of the small differences in the various kinase binding sites to improve the selectivity of the inhibition (Parang and Cole 2002).

The availability of an increasing number of three-dimensional structures of inhibitor/kinases complexes are helping in clarifying the atomic basis for the different selectivity and potency (Toledo et al. 1999). These crystal structures are often the starting point for the optimization of additional analogs through rational drug design approaches. A representative case is that of CDK2, whose crystal structures in complex with a number of key inhibitors has been used to explain the observed structure-activity relationships within the compound series and to guide the design of more potent inhibitors (Gray et al. 1998; Davis et al. 2001; Hardcastle et al. 2002). Nowadays, the first step in the research of a new promising chemical scaffolds is often the running of a virtual screen of a huge amount of chemical compounds utilizing as a target the three-dimensional structure of the protein kinase in complex with some inhibitor (Stahura et al. 1999). A similar approach has been successful in the case of different tyrosine kinases such as epidermal growth factor receptor (EGFR)/ErbB2 kinases, Flt-1 kinase, and Abl kinase (Traxler et al. 2001).

Probably the most exciting result in the field of kinase inhibitors concerns chronic myelogenous leukemia (CML), due to a translocation involving chromosome 8 and 22 that generates the BCR-Abl fusion protein. In normal cells, c-Abl is found in the nucleus as a negative regulator of cell growth, while BCR-Abl behaves as a fully cytoplasmic intrinsically active Tyr-kinase, giving rise to the development of this cancer. Recently, a very potent and selective inhibitor of BCR-Abl, Gleevec (or Glivec or STI-571 or CPG 57148B), a phenylamino-pyrimidine derivative, has come into the market in the treatment of CML, with very low or even null side effects. An important step in the comprehension of the mechanism of inhibition has been the elucidation of the crystal structure of the Abl domain in complex with STI-571.

8 CK2 Inhibition

The oncogenic potential of CK2 and its involvement in virally mediated pathologies has led to an increasing number of studies aimed at the discovery of selective inhibitors. These molecules could constitute not only lead compounds for drug development, but also useful tools for the clarification of the protein function in vivo.

An important aspect of CK2 that could simplify the drug design process is that this enzyme is always active and does not undergo the relevant conformational changes typical of most protein kinases. This circumstance allows us to overcome the difficulty that sometimes arises in correlating biochemical inhibition data, often obtained with activated kinases, with structural data derived from inhibitors bound to the inactive form of the enzyme. Moreover, it is not always possible to crystallize protein kinases in their active state. In the case of complexes with the inactive form of kinases, caution must be used in the straightforward interpretation of the inhibition data in terms of structural details that can represent a situation slightly but significantly different from that occurring in the biochemical experiments. However, there are examples, as with the Abl kinase, where the structure of the complex between an inhibitor, Gleevec in this case, bound to an inactive state of the enzyme could help in the interpretation of the biochemical data.

Two characteristics of CK2 must be considered in the development of highly specific inhibitors: (a) the active site can utilize both ATP and GTP as co-substrates and this feature is quite unique among kinases that usually are specific for ATP; (b) staurosporine is a well-known, potent inhibitor of protein kinases, with a really broad spectrum of activity, but it is only marginally efficient on CK2 (Meggio et al. 1995). These two features indicate that the catalytic site of CK2 has some unique characteristics that can be exploited to design inhibitors with a high degree of specificity. The determination of the three-dimensional structure of CK2 in complex with different inhibitors is an essential step in the elucidation of the critical properties of the active site.

In recent years we have determined the crystal structure of several complexes between the α -catalytic subunit of CK2 from Zea mays and ATP sitedirected inhibitors. In particular, we have studied four members of the anthraquinone-related compounds, one halogenated benzotriazole and an indoloquinazolinone derivative. To date, the best-characterized classes of CK2 inhibitor are the following: (a) hydroxylated polycyclic aromatic compounds with anthraquinone/xanthenone or fluorenone or flavone scaffolds; (b) halogenated benzimidazoles or benzotriazoles; (c) indole derivatives of quinazolinone.

9 Common Features of Inhibitor-CK2 Complexes

The close structural similarities between the Zea mays and the human α -catalytic subunits strongly support the decision to work with the maize enzyme in structural inhibition studies: this enzyme has a higher tendency to produce good diffracting crystals, and this is the reason why it has been preferred to the human enzyme.

Like the majority of the kinase inhibitors, those crystallized in complex with maize CK2 also bind to the catalytic site of the enzyme in the position normally occupied by an ATP (or GTP) molecule that acts as phosphate do-nor (Fig. 3).

Since the crystal structure of the apo-form of CK2 is known (Battistutta et al. 2001), an evaluation of the effect of the different inhibitors on the enzyme structure is possible.

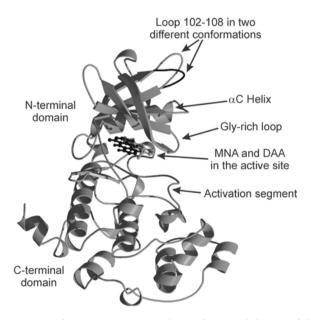


Fig. 3 Crystal structure of maize CK2 in complex with two inhibitors of the anthraquinone family. The positions of MNA and DAA in the active site are indicated. Note the different conformations of loop 102–108 (see text for discussion)

In general, the structure of the protein is poorly affected by the binding of a small molecule, either a co-substrate, such as ATP or GTP, or an inhibitor. The C-terminal lobe of the protein, conventionally considered starting with asparagine 118 and extending to the C-terminal end, is practically unaffected by the formation of the complex. The superposition of the different structures indicates a remarkable rigidity, with the exception of few residues facing the catalytic site, mainly Asn118, His160, and Met163. The N-terminal lobe shows a higher degree of flexibility than the C-terminal one, although the changes are in any case limited. The β -strands present in the N-terminal domain have the ability to "fine tune" their position to better accommodate ligands with different properties. Near the active site, in the glycine-rich loop and in the hinge region, some residues can be found with different conformations depending on the bound molecule. As reported in the following sections, the Gly-rich loop backbone has shown a significant flexibility, as indicated by the ability to move "up" or "down" depending on the ligand (Fig. 4). A functional relevance was recently attributed to this flexibility noted in the inhibitors/CK2 complexes. It has been suggested that in the holoenzyme the β -subunit can contribute to block and stabilize this region from behind the catalytic subunit (back-stabilization) and that this can destabilize a possible closed inactive conformation of the CK2 activation segment (Ermakova et al. 2003).

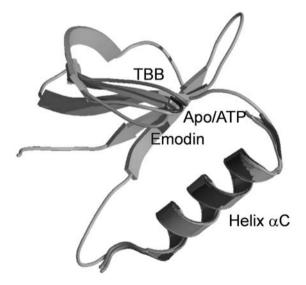


Fig. 4 Superposition of the Gly-rich zone and the α C helix of CK2 in the Apo form and in complex with emodin, tetrabromo-2-benzotriazolo (*TBB*), and ATP. The position of the α C-helix is very well conserved in all CK2 structures. The Gly-rich loop shows a higher degree of variation depending on the occupancy of the ATP-binding site

In many kinases the presence of an inhibitor in the active site alters the conformation of the flexible Gly-rich loop, often by means of an interaction with the aromatic residue preceding the third glycine (Huse and Kuriyan 2002). In CK2 a similar interaction has never been observed.

Comparing the crystal structures of several CK2 α -subunits from Zea mays, changes were noted in the external loop spanning residues 102–108, found in two different conformations (Fig. 3 and Table I; De Moliner et al. 2003). These changes have been attributed to crystallization and/or freezing condition rather than to ligand-binding effects, since they are reflected on small but significant variations in some parameters of the crystallographic unit cell. The recent independent determination of the crystallographic structures of the human α -subunit alone and of a mutant showed this region in the same conformation, defined as "closed," while in the holoenzyme the "open" conformation has been detected. Whether this variation is only a crystallographic artifact, as formerly suggested (De Moliner et al. 2003), or has a functional relevance, as subsequently proposed (Ermakova et al. 2003), remains an open question.

The position of the helix in the N-terminal domain, helix αC , is particularly well conserved in all the three-dimensional structures of CK2 known to date (Fig. 4). As reported above, the correct position of this helix is one of the most striking properties of active protein kinases. The maintenance of the position of helix αC is therefore well in accordance with the notion of the constitutive activity of CK2, as well as the overall rigidity of the enzyme.

The stretch of residues whose conformation is less characterized is that spanning from Leu70 to Lys76, just before and at the beginning of helix α C. The electron density of these residues is always worse defined compared to that of the remaining part of the protein, especially in the side chains, indicating an intrinsic flexibility of this zone. In spite of that, the orientation of helix α C is not affected, as reported above. As the relatively mobile 70–76 segment is considered involved in the substrate recognition, this flexibility is probably related to the lack of a phosphorylatable amino acid stretch that can eventually interact and block these residues.

The conformation of the Met163 side chain, a residue distinctive of CK2 located in the middle of the active site, is significantly affected by the presence of both inhibitors and co-substrate. In apo-CK2 α , the side chain of Met163 stretches out and the terminal methyl group points into the cavity. When the catalytic pocket is occupied, the side chain is folded away from the center of the cleft, leaving free space for the ligand.

10 Anthraquinones Inhibitors

Anthraquinones are compounds able to interact with the nucleotide-binding sites of enzymes such as dehydrogenases, kinases, and ATPases, due to their structural similarities with adenine nucleotides such as ATP, adenosine diphosphate (ADP), and nicotinamide adenine dinucleotide (NAD). Anthraquinones and xanthones from natural sources have several potential therapeutic applications, for instance as antiviral, antimicrobial, or anti-cancer drugs (Ali et al. 2000). The extended planar structure of these compounds possibly makes them cytotoxic in that they are able to act as DNA intercalators. Even if that makes questionable the employment of this class of compounds in clinical practice, one might take advantage of them as chemical tools to elucidate the different cellular functions and regulations of CK2 for the optimization of highly specific and selective inhibitors.

Several anthraquinones and related xanthones have been tested with in vitro phosphorylation assays as potential inhibitors for CK2 (Sarno et al. 2002). Emodin (1,3,8-trihydroxy-6-methyl-antraquinone), previously reported as a tyrosine kinase inhibitor (Chan et al. 1993), was the first member of the anthraquinone family to be discovered that was also able to inhibit CK2 (Yim et al. 1999). The chemical formula and the inhibition potency (inhibitory constant, K_i) for emodin as well for other anthraquinones is reported in Fig. 5. Starting from this parent compound, a large number of anthraquinones and xanthone derivatives have been screened on CK2, allowing the identification of some interesting compounds with lower IC₅₀ (or K_i) values. The most promising ones have been submitted to crystallization trials, and for those that have generated good diffracting crystals (Fig. 5), the

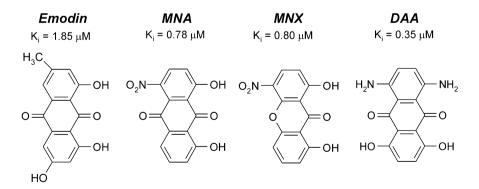


Fig. 5 Chemical formulae and inhibition potencies for the four anthraquinones crystallized in complex with CK2. *Emodin*, 1,3,8-trihydroxy-6-methyl-antraquinone; *MNA*, 1,8dihydroxy-4-nitro-anthraquinone; *MNX*, 1,8-dihydroxy-4-nitro-xanthen-9-one; *DAA*, 1,4diamino-5,8-dihydroxy-anthraquinone

three-dimensional structures have been determined. The inhibitory constant K_i for the four compounds crystallized is in the micromolar range, from 1.85 μ M for emodin to 0.35 μ M for DAA.

Emodin is the first anthraquinone derivative crystallized in complex with Zea mays CK2 as an ATP site-directed competitive inhibitor. This natural compound is extracted from the rhizomes of *Rheum palmatum* and is used as anti-inflammatory and anti-cancer drug, especially in the Asian continent (Yim et al. 1999). Emodin has an inhibitory constant (K_i) of 1.85 μ M for CK2, while it is poorly effective on other Ser/Thr protein kinases. This compound is able to inhibit also some Tyr-kinases, although with a lower efficiency (Jayasuriya et al. 1992). For instance, the receptor Tyr-kinase Her-2 neu is inhibited with a K_i value of 21 μ M (Zhang et al. 1998), an order of magnitude higher than that for CK2.

Crystals of the emodin/CK2 complex were obtained by the soaking method (Battistutta et al. 2000). As emodin is poorly soluble in aqueous media, crystals of the maize CK2 catalytic subunit in the apo-form have been soaked for several hours with a saturated solution of emodin, which results in red for the presence of the inhibitor. The formation of the complex was evident because, within a few hours, the red coloring concentrated in the crystals, whereas the surrounding solution changed to pale orange. These crystals are isomorphous with those of maize CK2 grown in the presence of co-substrates ATP and GTP and also with those of the other anthraquinones complexes obtained with co-crystallization procedures (see below); all belongs to the C2 space group, with cell parameters reported in Table 1.

The co-substrate-competitive inhibitory activity of emodin is due to its ability to bind to the active site of CK2. Although the binding of the inhibitor poorly influences the overall architecture of the protein that remains in a structurally (but obviously not functional) active state, some regions of the catalytic subunit undergo appreciable structural changes, especially in the N-terminal lobe. The rms deviation for the 112 α -carbon atoms of the N-ter-

CK2 complexes	Space group	a, b, c (Å)	α, β, γ (°)	Resolution (Å)
Apo-CK2	C2	142.8, 58.5, 45.1 ^a	90.0, 102.6, 90.0 ^a	2.10
Emodin	C2	143.1, 52.1, 44.9 ^b	90.0, 99.3, 90.0 ^b	2.63
MNA	C2	142.1, 60.6, 45.0 ^a	90.0, 102.8, 90.0 ^a	2.00
MNX	C2	143.0, 51.7, 44.7 ^b	90.0, 99.3, 90.0 ^b	1.79
DAA	C2	143.1, 51.8, 44.7 ^b	90.0, 99.7, 90.0 ^b	1.70
TBB	P1	48.3, 55.9, 60.3	89.8, 102.5, 99.3	2.19
IQA	C2	141.5, 60.3, 44.4 ^a	90.0, 102.6, 90.0 ^a	1.68

Table 1 Space group, unit cell parameters, and maximum resolution of diffraction for crystals of CK2 in the Apo form and in complex with inhibitors described in this chapter

^{a,b} Different cell parameters corresponding to the two conformations of loop 102–108 (see text for discussion).

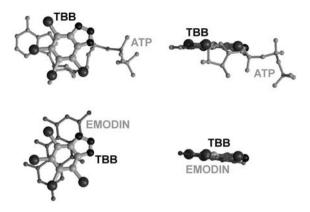


Fig. 6 Superposition of TBB and ATP (*upper panel*) and emodin (*lower panel*) in the active site of CK2. A "top" view" on the *left* and a "side" view (90° rotation) on the *right* are shown. TBB is chosen as reference

minal lobe, from positions 7 to 118, is 1.29 Å, while the rms deviation drops to 0.40 Å for the 215 α -carbon atoms of the C-terminal domains, from position 119 to 333.

The two most variable regions are the Gly-rich loop from residue 58 to 63 and the exposed loop from residue 102 to 108. The electron density of this second long loop is poorly defined, indicating a remarkable flexibility of this zone. For other anthraquinone complexes, crystals of better diffracting quality and higher resolution have been obtained with co-crystallization methods. In these cases, the electron density of the loop 102–108 is better defined, allowing the detection of the two possible conformations of this loop, the "open" and the "closed" ones (Fig. 3).

The emodin inhibitor is inserted into the active site cavity deeper than the ATP or GTP molecules, with only two out of three rings roughly superimposable to the nucleotide base (Fig. 6). As a result, the inhibitor occupies a hydrophobic zone of the CK2 active site that shows a certain degree of variability among protein kinases (Scapin 2002), and this can be responsible for the limited selectivity of this inhibitor.

Among the anthraquinone derivatives studied so far, emodin causes the larger variations in the conformation of the residues belonging to the active site. Around the binding site, significant differences in the position of amino acid Asn118, of the side chain of His160, and of the triplet Arg47-Gly48-Lys49 have been detected (Fig. 7). In particular, the backbone segment from Arg47 to Lys49, which is part of the Gly-rich loop, is found shifted toward the interior of the active site, in a position that entraps emodin inside. Emodin is kept in the active site between the two lobes of the protein mainly by hydrophobic contacts involving residues Val45, Val53, Ile66, Leu85, Val95, Leu111, Phe113, Val116, Met163, and Ile174. The release of the inhibitor

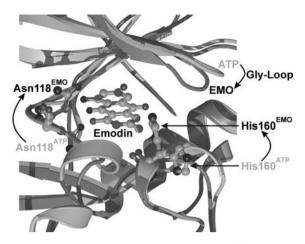


Fig. 7 Movements caused by emodin (*EMO*) binding. The different positions of Asn118, Gly-rich loop, and His160 of CK2 in complex with ATP (*gray labels*) and with emodin (*black labels*) are illustrated. A portion of the α C-helix can be distinguished behind His160

from the cavity is hampered both by the 46–50 segment and by residues His160 and Asn118 (Fig. 7), whose α -carbon moves about 1.2 Å from its original position. The orientation of the side chain of Asn118 contributes to the enclosure of the cavity, as well as the imidazole ring of His160, which is able to form a new H-bond with the carbonyl oxygen of Arg47.

The presence of hydroxyls at positions 6 and 8 of emodin seems relevant for an optimal inhibitory potential (Jayasuriya et al. 1992). In our structure, no clear polar interactions have been found between the inhibitor and the protein. The hydroxyl group in position 6 is 3.40 Å away from the backbone carbonyl of Val45, 3.70 Å from the amide nitrogen of Arg47, and 3.98 Å from the imidazole nitrogen of His160, while that in position 8 is 3.99 Å away from the side chain carbonyl of Asn118. These interactions are too loose to be considered hydrogen bond-like, but the relatively low resolution of 2.63 Å of the model does not completely rule out the possibility of polar interactions between the inhibitor and the protein. In this respect, it can be relevant to notice that emodin does not completely fill in the cavity, whose actual volume is slightly larger than that of the inhibitor. The quality of the electron density of emodin is to some extent poorer than that of the surrounding residues, suggesting that emodin could maintain some degree of freedom once encapsulated into the pocket. The higher mean value of the emodin thermal B factor of 58 \AA^2 compared to that of the backbone atoms of the enzyme of 50 \AA^2 is consistent with these observations. On the other hand, another possible or complementary explanation of the effect of the hydroxyl functions is that they increase the solubility of emodin in an aqueous environment.

Emodin shows a slightly higher inhibition constant against CK1 than against CK2. This observation can be explained with the conservation of the main requirements for the inhibitor binding in the two kinases. On the other hand, cAMP-dependent protein kinase (cAPK) is inhibited by emodin with a constant two orders of magnitude higher, and this corresponds to a higher variation in the active sites characteristics. Residues Val66, Ile174, and Phe113 in CK2 are replaced by Ala70, Thr183, and Met120 in cAPK, with the latter two closer together (only 3.80 Å apart). A comparison of the molecular models of CK2 and cAPK indicates that emodin cannot interact in the same way with the two proteins, unless a large rearrangement of residues Thr183 and Met12 of cAPK takes place.

Crystals of CK2 complexes with other anthraquinones, namely MNA (a mono-nitro derivative, 1,8-dihydroxy-4-nitro-anthraquinone), MNX (a mono-nitro xanthenone, 1,8-dihydroxy-4-nitro-xanthen-9-one), and DAA, (a di-amino derivative, 1,4-diamino-5,8-dihydroxy-anthraquinone), have been obtained by co-crystallization techniques. This approach has allowed for the achievement of better resolutions (up to 1.70 Å) with respect to soaking methods used in the case of emodin and TBB (Table 1). The poor solubility in water of the inhibitors has been overcome by dissolving them at high concentration in dimethylsulfoxide (DMSO) and then incubating the protein with the solution obtained for a final maximum concentration of the organic solvent equal to 5%.

Among these three inhibitors, MNX is the one that causes the greatest variation in the N-terminal domain of the enzyme, at the level of the backbone from residues 72 to 75 and of the Gly-rich loop (residues 45–51). The latter "collapses" into the co-substrate binding cavity, in a manner similar to that displayed by the emodin complex. In the case of MNX, this displacement is not coupled with a rotation of the His160 and Asn118 side chains.

MNA and MNX, whose positions in the active site are fully superimposable (Fig. 8), orient the nitro group toward the hinge region, with the result that the hydroxyl groups can make contacts with Lys68 (Fig. 9). Besides hydrophobic and van der Waals interactions, in comparison with emodin and TBB (see next paragraph), MNX and MNA are able to form further polar interactions with the active site of the enzyme. These additional interactions contribute both to increase the affinity for the enzyme, as indicated by the lower K_i values, and to orient the molecules in a different way inside the active site.

In the MNA/CK2 complex, the nitro group of MNA is found lying in a plane roughly perpendicular to that of the aromatic rings (Fig. 8), as expected for the steric hindrance of the adjacent carbonyl group (*ortho* effect). In the crystal, MNA is present in the active site in a double conformation, with the nitro group oriented toward the external portion of the ATP-binding cavity in the higher occupancy conformation (67%). MNA forms four hydrogen bonds: the two hydroxyl groups and the carbonyl oxygen located on the

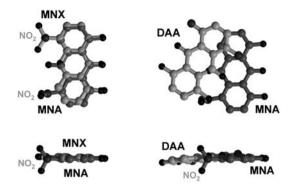


Fig. 8 Superposition of the three anthraquinones MNA (taken as reference), MNX, and DAA in the active site of CK2. Note that the nitro group of MNA is not co-planar with the aromatic rings as in the case of MNX

same side of the inhibitor interact with side chains of Asp175, Lys68, and a solvent molecule (Fig. 9). Similarly to the cases of the MNX/CK2 and DAA/CK2 complexes, this solvent molecule interacts also with Glu81 and Trp176.

In MNX, the central carbonyl function is absent and, therefore, in the crystal structure the nitro group is co-planar with the aromatic plane

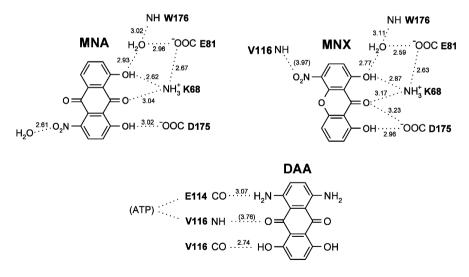


Fig. 9 Polar interactions between CK2 and anthraquinone inhibitors. Hydrogen bonds and salt bridges are indicated by *dotted lines*; distances are reported in Ångstroms. For DAA, the distance to the NH of Val116 is reported for comparison with ATP binding (ATP is hydrogen bound to backbone CO of Glu114 and backbone NH of Val116 in the hinge region)

(Fig. 8). MNX interacts with the protein in a way similar to MNA, but it enters the active site in one single orientation, with the nitro group in the deeper position inside the cavity.

The importance of the nitro function in MNA and MNX is outlined by the observation that, if missing as in 1,8-dihydroxy-anthraquinone and 1,8-dihydroxy-xanthen-9-one, the inhibitory constants (K_i) rise to values higher than 40 μ M. However, in our models there are no direct chemical interactions between the nitro groups and the protein. This leads to the hypothesis that the effect of the NO₂ group is related to the electron-withdrawing property of this substituent, which is able to increase the dissociation constant of the phenolic hydroxyl group in the *para* position. As a result, this becomes more acidic, as confirmed both by theoretical analyses and experimental data. The resulting partially negatively charged oxygen can provide a better anchor for Lys68, with which it is interacting at distances typical for hydrogen bonds formation.

The third inhibitor of the series, DAA, that lacks the nitro group, binds to the active site in a different way if compared to MNA and MNX. DAA prefers a position inside the cleft near the hinge region, where it can interact with backbone carbonyls of Glu114 and Val116 via two hydrogen bonds (Fig. 9). This particular mode of interaction confers to DAA one of the highest inhibition potency (K_i value of 0.35 µM) among the anthraquinones studied so far.

Taken together, the four crystal structures of CK2 in complex with anthraquinone derivatives revealed three different modes of binding to the ATPbinding site. In spite of their common scaffold, anthraquinones can anchor to the hinge region, as DAA does in a way similar to the adenine moiety of ATP, or they can interact with Lys68 and Asp175, although with different orientations (MNA and MNX), or, finally, they can sit deeply inside the cavity with no strong polar interactions with the protein (emodin).

11 Tetrabromo-2-Benzotriazole

4,5,6,7-tetrabromo-benzotriazole (TBB or tetrabromo-2-azabenzimidazole) is a CK2 inhibitor developed starting from the 5,6-dichloro-1-(β -d-ribofuranosyl)benzimidazole (DRB) parent molecule. DRB was found inhibiting CK2 with a relatively high inhibitory constant, (K_i =24 μ M) and a rather low specificity (Zandomeni et al. 1986). After several modifications (Fig. 10), namely the replacement of two chlorine atoms with four bromine ones, the depletion of the sugar moiety, and the substitution of the imidazole ring with a triazole, the final compound not only shows a lower K_i (0.2–0.6 μ M), but also a higher selectivity for CK2 (Szyszka et al. 1995; Shugar 1999). At variance with the precursor, TBB is also able to discriminate between CK1

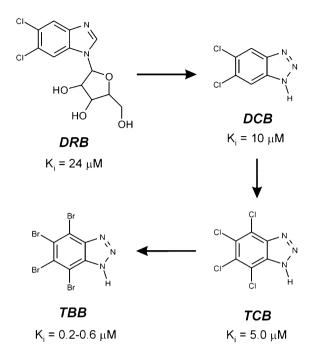


Fig. 10 Scheme of the chemical modifications that have led to the development of TBB starting from DRB (see text for details). The K_i for the four compounds is reported

and CK2 (Sarno et al. 2001) and is one of the most selective CK2 inhibitor now known.

Crystals of the complex CK2/TBB belong to the P1 space group (Battistutta et al. 2001), while all crystals of the catalytic subunit of CK2 from *Zea mays*, either in the apo form or in complex with co-substrates or inhibitors, belong to space group C2 (Table 1). The reason for this variation in the crystal parameters is not fully understood.

As in the case of the anthraquinones, the N-terminus domain shows a more noticeable variation than the structurally more conserved C-terminus one, with the remarkable exception of helix α C. Again, regions from residue 102 to 108 and from 70 to 74, immediately upward the α C helix, show a less defined electron density and a higher flexibility, as indicated by the higher B-factor values. TBB binds to the active site essentially by means of hydrophobic interactions involving residues Val45, Val53, Ile66, Val95, Phe113, Val116, Met163, and Ile174. Due to the bulkiness of the four bromine atoms, TBB fills almost perfectly the active site cleft (Fig. 11) and this appears to be the main reason for the potency and selectivity of the inhibitor. This hypothesis is corroborated by the lower activity of two TBB analogs, where bromine atoms are substituted by the less bulky chlorines. The inhibitory constants of tetrachlorobenzotriazole (TCB) and dichlorobenzotriazole

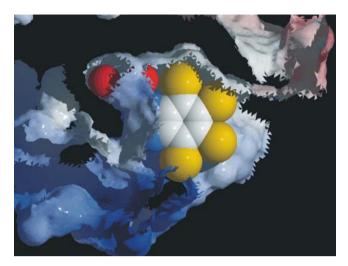


Fig. 11 Full spheres (CPK) model of TBB in the active site of CK2. A portion of the surface of the ATP-binding site is shown colored according to the electrostatic potential (positive charges in *blue*, negative charges in *red*). TBB atoms colors: carbon in *white*, nitrogen in *blue*, bromines in *yellow*. The two water molecules that connect TBB to Trp176 and Glu81 are represented as *red spheres*

(DCB) are 5 and 10 μ M respectively (Fig. 10), one order of magnitude higher than that of TBB (Shugar 1999).

Nitrogen N1 of TBB is linked to $O\epsilon^2$ of Glu81 via two water molecules that make a bridge between the two atoms (Figs. 11 and 12). This is the only po-

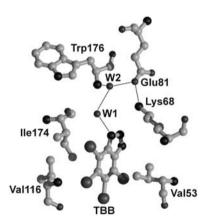


Fig. 12 Ball-and-stick models of TBB and some of the interacting residues. Polar interactions are indicated by *continuous lines*. Water molecules (W1 and W2) are represented as *isolated spheres*. Carbons in *gray*, nitrogens, oxygens, and bromines (*larger spheres*) in *black*. Residues 53, 116, 174 are part of the hydrophobic surface that surrounds TBB

lar interaction present in the complex. The structural importance of one of these two water molecules (W2 in Fig. 12) is testified by its presence in almost all the CK2 crystal structures now determined. This solvent molecule is tightly bound to the deeper portion of the active site where polar side chains are present, for instance the well-conserved Lys68 and Glu81. As seen for several inhibitors, it can be exploited by ligands as hydrogen bond anchoring sites. Two other important water molecules have been identified in the ATP and GTP complexes of CK2 (Niefind et al. 1999) as responsible for the unique "dual-co-substrate specificity" of CK2. All these water molecules seem to play a specific role in modulating the ligand/protein interaction inside the ATP-binding site.

In the CK2/TBB complex, histidine-160 side chain adopts the same orientation as in the presence of emodin. In the presence of TBB, however, the Gly-rich loop is shifted away from His160, probably owing to the large size of the bromines, with the consequence that the H-bond between the His160 side chain and Arg47 backbone cannot take place. This highlights once more the flexibility of the glycine loop, capable to adjust itself to the type of ligand bound in the cavity. In fact, the Gly-rich loop has a very similar conformation in the apo-CK2 and when the enzyme is bound to ATP or GTP, whereas in the presence of emodin and TBB it moves toward or away from the N-terminal lobe, respectively (Fig. 4).

In the attempt to rationalize the selectivity of TBB toward CK2 on a wide panel of different kinases (Sarno et al. 2001), the active sites of the kinases whose structure is known have been compared to that of CK2. From this analysis it emerges that CK2 bears an ATP-binding site invariably smaller in size, due to some characteristic bulky side chains which in CK2 reduce the space available to cofactors or inhibitors. These typical residues are Ile(Val)66, Met163, and Ile174, which in most protein kinases are replaced with less bulky amino acids, namely alanine versus Ile66, leucine or valine versus Met163, alanine, threonine, or leucine versus Ile174. This suggests that a perfect fit in shape between TBB and the active site is crucial for the selectivity and is also supported by the results obtained with a mutant where Ile(Val)66 has been substituted by an alanine. Such a mutant is less susceptible to the inhibitory activity of TBB.

The smaller dimension of the active cleft in CK2 with respect to other kinases can also be the reason for the unusually modest sensitivity to staurosporine (Sarno et al. 2002), whose large molecular size may hamper the entrance into the active site of CK2.

12 Indoloquinazolinones

To identify new inhibitors, a virtual screening analysis among the in-house collection of chemical compounds has recently been performed by researchers of Novartis Pharma against a human CK2 three-dimensional model (Vangrevelinghe et al. 2003). This analysis has led to the discovery of a new class of potent and selective inhibitors of CK2, the indoloquinazolinones. The most promising compound, IQA [(5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl)acetic acid], with a K_i of 0.17 μ M, has been co-crystallized in complex with CK2 (Table 1) and the final three-dimensional structure at 1.68 Å resolution has been recently reported (Sarno et al. 2003).

IQA lays inside the catalytic pocket of CK2 on the same plane occupied by the ATP purine moiety. The hydrophobic side of IQA faces the hinge region of the protein, while the polar side is oriented toward Lys68. IQA is found in the ATP-binding site in two different orientations, differing for a rotation of the acetate function (Fig. 13). The two alternative conformations have been refined with final occupancies of 50%. In the first conformation, the carboxylic function makes an internal hydrogen bond with the lactam nitrogen, closing a six-member ring, and interacts with the hydroxyl oxygen of Ser51. This conformation is largely favored in solution (Fig. 14), as confirmed by nuclear magnetic resonance (NMR) spectroscopy analysis. Therefore, it has to be assumed that the second conformation is stabilized when bound to the CK2 active site, where it is found as abundant as the first one. In fact, in the second conformation the carboxylate group is involved in

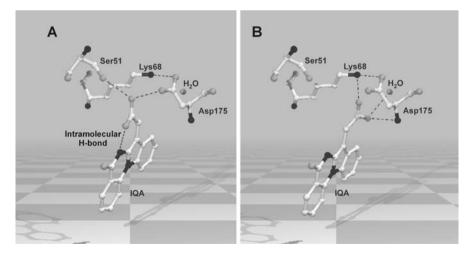


Fig. 13A, B Polar interactions of IQA with Ser51, Lys68, and Asp175 of CK2. IQA binds to CK2 in two conformations, shown in A and B

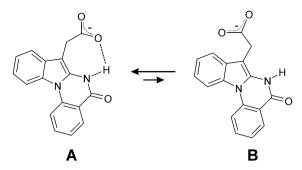


Fig. 14A, B Equilibrium between the two conformations of IQA present in solution. As evidenced by nuclear magnetic resonance (NMR) analysis, the species with the internal hydrogen bond is predominant in solution (species A on the *left*)

three hydrogen bonds, one with the amine function of Lys68, one with the backbone nitrogen of Asp175, and one with a water molecule in turn connected to the backbone amide of Trp176 (Fig. 13). This water molecule is the same found in all CK2 structures solved to date, with the exception of the emodin complex, as already mentioned.

For both conformations, the major contribution to the binding comes from the hydrophobic interaction with non-polar residues in the binding site, i.e., Val45, Val53, Ile66, Lys68, Val95, Phe113, Val116, Met163, and Ile174. In this respect, it is remarkable that the total buried surface upon inhibitor binding is quite large, about 730 Å². The importance of the hydrophobic interactions in the binding of IQA is supported by the considerable decrease of the inhibitory efficiency observed in the cases of Val66Ala and Ile174Ala mutants. In fact, mutation of Ser51 with a glycine does not significantly affect the K_i value. This is in agreement with the observation that the two different orientations of IQA bind with similar affinity, since they share the hydrophobic contacts, while they differ in the interaction with Ser51.

The best inhibitor of CK2 discovered so far, IQA, albeit more potent and selective than those previously available, is not yet totally specific for the enzyme. With the increasing number of three-dimensional structures of inhibitors/CK2 complexes, it should be possible to improve the selectivity of inhibitors using a structure-based drug design method. Anyhow, this task is not straightforward, since we have seen from the previous examples that compounds extremely similar to one another, like MNA, MNX, DAA, and emodin, bind to the active site of the enzyme in a significantly different way. In practice, a limited modification of the structure of the inhibitor, for example the addition of a substituent with the aim of increasing the number of H-bond interactions (like the addition of an OH or NH_2 group), often changes the electronic properties of the inhibitor, modifying its binding mode. In the case of CK2 inhibitors, this is probably worsened by the rela-

tively limited complexity of the small molecules used. A more extended surface of interaction between the inhibitor and the enzyme could help not only to decrease the K_i , but also to stabilize its binding mode.

Another point about computer-aided drug design has to be stressed. In the in silico procedure for the identification and characterization of the indoloquinazolinone derivatives, such as CK2 inhibitors, a model of IQA bound to the enzyme has been proposed (Vangrevelinghe et al. 2003). In this model, the acetate group of IQA faces the hinge region and interacts with arginine-43, with a orientation opposite to that found in the experimental crystal structure. In this case, it comes out that while virtual screening protocols could usefully select some interesting compounds among a large database, the docking procedures have been unable to correctly position the inhibitor in the enzyme active site. For the correct detailed description of the interactions between IQA and CK2, the determination of the crystal structure of the complex was an unavoidable step.

13 Concluding Remarks

In this chapter we have described the binding modes of some CK2 inhibitors that target the ATP-binding site. These inhibitors belong to different chemical families and have inhibitory constants in the low micromolar range. Two of them, namely TBB and IQA, display a significant selectivity among panels of tens of different kinases, and can be considered an encouraging starting point for the development of compounds of potential pharmacological interest.

For all the inhibitors here described, the main energetic contribution to the binding is attributed to hydrophobic interaction with the apolar surface region of the CK2 binding cleft. In this respect, the shape and the reduced dimension of the CK2 active site in comparison with other kinases are essential in explaining the selectivity of these inhibitors as well as the anomalous low potency of staurosporine. In particular, isoleucine/valine-66 and isoleucine-174, residues unique to CK2, play an important role as demonstrated by mutagenesis studies. The aromatic nature of the inhibitors can be considered a key characteristic of these compounds, providing the main energetic contribution to the binding.

In general, the ATP-binding sites of kinases present both polar and hydrophobic portions. While the characteristics of the polar moiety are quite conserved among the family, as, for instance, some key residues in the hinge regions, in the phosphate anchor region, or other residues like lysine-68, glutamate-81, and aspartate-175 (CK2 numbering), the hydrophobic ones have a higher degree of variability in the amino acid composition. Consequently, the selectivity is ensured by targeting the hydrophobic portion of the binding site while the presence of polar interactions (hydrogen bonds, salt bridges) enhances the potency of the inhibitor. The inhibitors here described can be considered more selective than potent, and this is in agreement with the above considerations in that they bind to the enzyme mainly through hydrophobic interactions. As noticed for the four anthraquinones, the presence of polar interactions is essential to increase the binding potency; in this respect, the interactions with the hinge region seem more effective (see the case of DAA in comparison with emodin). To produce inhibitors in the nanomolar range, as would be desirable, our aim is now concentrated in the increase of the number and strength of the polar interactions between the inhibitors and the enzyme, without depleting the hydrophobic interactions. Other important points are the increase of the solubility of these compounds in water and the analysis of their potential pharmacological properties.

The estimate of the selectivity of a kinase inhibitor is always a difficult task; in fact, while the number of kinases has been estimated around 500 from the human genome, the enzymes really available for inhibition assays are only a fraction of that (around some tens). The development of enzyme mutants resistant to the inhibitory activity of a specific compound but with a catalytic activity similar to the wild-type protein [i.e., mutants Ile66Ala and Ile174Ala for CK2 (Sarno et al. 2002)] can be very useful in the analysis of its selectivity. In fact, the recovery of the original kinase activity by means of such mutants in the presence of the specific inhibitor in in vivo assays can be considered a validation of the involvement of just that kinase in the process under study.

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Aminoglycoside Kinases and Antibiotic Resistance

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Abstract The evolution of antibiotic-resistant bacteria represents a serious public health concern. The appearance of strains with resistance to multiple antibiotics threatens to render some infections untreatable by existing drugs. As a result, there is considerable interest in understanding the mechanisms of antibiotic resistance and in identifying ways in which antibiotic resistance can be overcome. Aminoglycoside antibiotics are broad-spectrum bactericidal compounds that are commonly used in the treatment of serious nosocomial infections. They exert their activity by binding to the A-site of the bacterial 30S ribosomal subunit where they impair the fidelity of protein translation. A number of bacterial strains have developed resistance to many aminoglycosides as a result of their acquisition of aminoglycoside-modifying enzymes that inactivate the antibiotic by reducing its affinity for the bacterial ribosome. These modifying enzymes can be classified

into three groups according to the identity of the group used to modify the antibiotic substrate-aminoglycoside acetyltransferases, aminoglycoside phosphotransferases (kinases) and aminoglycoside nucleotidyltransferases. One of the best-understood aminoglycoside-modifying enzymes is aminoglycoside 3'-phosphotransferase type IIIa [APH (3')-IIIa]. This enzyme catalyses the transfer of a phosphate moiety from ATP to a range of aminoglycoside substrates. Surprisingly, when the three-dimensional atomic structure of APH (3')-IIIa was determined, it was found to possess striking similarity to eukaryotic protein kinases. Strategies to overcome resistance to aminoglycoside antibiotics are multifaceted and include: (1) novel aminoglycosides that are either not modified, or have low affinity for modifying enzymes, (2) mechanism-based inhibitors that exploit the enzyme's activity, (3) inhibitors of eukaryotic protein kinases that bind in the nucleotidebinding pocket, (4) bridged molecules that interact with both the nucleotide and aminoglycoside binding sites and (5) cationic peptides that resemble protein kinase inhibitors. By pursuing these leads, it is hoped that compounds will be developed that will allow aminoglycoside antibiotics to remain useful components of the physician's armamentarium.

Keywords Aminoglycoside · Protein kinase · Antibiotic resistance · Inhibitor design

1 Introduction

Advances in the development of antibiotics are being compromised by bacterial populations exhibiting multiple antibiotic resistance and a number of antibiotics have been rendered clinically obsolete. The development of multidrug-resistant strains of bacteria creates the potential for epidemics of infections that are either untreatable or which can only be treated by one or a few antibiotics.

Aminoglycosides are a class of antibiotics used in the treatment of serious infections caused by gram-negative organisms. Some infections that resist treatment by other antibiotics have retained susceptibility to one or more aminoglycosides. However, the clinical effectiveness of this class of antibiotics has been seriously compromised by the emergence of strains of bacteria that are resistant to their effects.

2 Aminoglycosides and Aminoglycoside Resistance

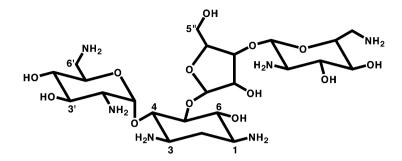
2.1 Aminoglycoside Antibiotics

The first aminoglycoside, streptomycin, was isolated from the soil bacterium *Streptomyces griseus* in 1944 (Schatz et al. 1944). Streptomycin proved to be the first successful drug against *Mycobacterium tuberculosis* and became

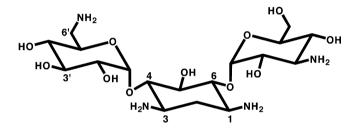
widely popular in the 1940s and 1950s. The impact of streptomycin was of such significance that Selman A. Waksman was awarded the Nobel Prize in Medicine in 1952 for its discovery. While streptomycin continues to be an integral part of modern chemotherapy for tuberculosis since its clinical introduction over 50 years ago, a variety of natural and semisynthetic aminoglycosides with broad antimicrobial spectra have also been discovered and developed. Despite their toxic effects on the kidney and the inner ear (Forge and Schacht 2000), aminoglycosides are among the most commonly used antibiotics due to their low cost and high efficacy against both grampositive and gram-negative bacteria, and in some cases, protozoal infections (Berman and Fleckenstein 1991).

From a chemical perspective, aminoglycosides are a group of structurally diverse, water soluble, polycationic molecules. They contain an aminocyclitol nucleus and two or three aminosugar rings linked to the nucleus via glycosidic bonds. They can be grouped into two main categories based on the structure of the central aminocyclitol ring. The first group, which includes streptomycin, contains a streptidine derivative; the second, larger group, which includes neomycin and kanamycin, contains a 2-deoxystreptamine ring derivatized at either the 4- and 5-positions or the 4- and 6-positions (Fig. 1). Conventionally, the numbering of the 6-aminohexose ring linked to the 4-position of the 2-deoxystreptamine is designated by prime ('), and the pentose or hexose ring linked to the 5- or 6-position is denoted by double prime ('').

Unlike many other antibiotics, aminoglycosides are bactericidal compounds. The primary target of these drugs in the bacterial cell is the 30S ribosomal RNA, as shown by chemical footprinting experiments (Moazed and Noller 1987), and more recently, evidence from crystallographic studies (Carter et al. 2000). Nonetheless, some details concerning the uptake and action of aminoglycosides remain elusive. Existing evidence indicates that the first step of aminoglycoside uptake involves an energy-requiring transport across the cell membrane (Wright et al. 1998). Once inside the cell, the aminoglycosides bind to the A-site (the decoding site) of the 16S ribosomal RNA (Moazed and Noller 1987) and trigger certain conformational changes in the A-site that normally occur only when there is a correct interaction between cognate tRNA and mRNA (Pape et al. 2000; Ogle et al. 2002; Rodnina et al. 2002). As a result, the stability of the binding of near-cognate aminoacyl-tRNA to this site is increased and the ribosome is unable to discriminate between cognate and near- or non-cognate tRNA-mRNA complexes, and the production of defective proteins ensues. Subsequently, the faulty proteins are presumably inserted into the cytoplasmic membrane, leading to the loss of membrane integrity. Additional aminoglycosides are then rapidly transported across the damaged membrane, leading to the accumulation of the drug in the cytoplasm, saturation of all ribosomes and ultimately cell death (Wright et al. 1998).



Neomycin B (4,5-disubstituted 2-deoxystreptamine)



Kanamycin A (4,6-disubstituted 2-deoxystreptamine)

Fig. 1 Examples of two aminoglycoside antibiotics based on 2-deoxystreptamine. Neomycin B is derivatized at the 4- and 5-positions of the central 2-deoxystreptamine ring, while kanamycin A is derivatized a the 4- and 6-positions

2.2 Resistance to Aminoglycosides

There are three principal mechanisms of bacterial resistance to antibiotics. First, the bacteria can prevent accumulation of antibiotics inside the cell either via changes in membrane permeability or via efflux pumps that export the drug from the cell. Second, the bacteria could also alter, by mutation or chemical modification (Thompson et al. 1985; Cundliffe 1989) the target of the antibiotic (such as the ribosome) to preclude the effects of the antibiotic. Last, antibiotics that manage to enter the cell can be enzymatically modified such that they have reduced affinity for their target and, therefore, can no longer exert their antimicrobial effects (Llano-Sotelo et al. 2002). This last mechanism is the most prevalent means of resistance to aminoglycosides in

AAC		APH	АРН		ANT	
AAC(2')	Ia, Ib, Ic, Id, Ie	APH(3')	Ia, Ib, Ic	ANT(4')	Ia	
-	IIa, IIb	-	IIa, IIb	-	IIa	
AAC(6')	Ia-In, Ip-Ix, Iz	-	IIIa	ANT(6)	Ia	
-	IIa, IIb	-	IVa	ANT(9)	Ia	
AAC(1)	Ia	-	Va	-	Ib	
AAC(3)	Ia, Ib	-	VIa	ANT(2")	Ia	
-	IIa, IIb, IIc	-	VIIa	ANT(3")	Ia	
-	IIIa, IIIb, IIIc	APH(4)	Ia, Ib	-	-	
-	IVa	APH(6)	Ia, Ib, Ic, Id	-	-	
-	VIa	APH(9)	Ia, Ib	-	-	
-	VIIa	APH(2")	Ia, Ib, Ic, Id	-	-	
-	VIIIa	APH(3")	Ia, Ib	-	-	
-	IXa	APH(7'')	Ia	-	-	
-	Ха	-	-	-	-	

Table 1 Aminoglycoside-modifying enzymes

clinical isolates (Davies 1991). Three families of aminoglycoside-modifying enzymes have been identified. They are aminoglycoside *N*-acetyltransferases (AACs), aminoglycoside *O*-phosphotransferases (APHs) and aminoglycoside *O*-nucleotidyltransferases (ANTs). They render aminoglycoside antibiotics inactive by catalysing the transfer of an acetyl group (from acetyl CoA), a phosphate group or an adenyl group (both from ATP) to the aminoglycoside. Over 50 aminoglycoside-modifying enzymes have been identified (Table 1) and they are named using nomenclature proposed by Shaw (Shaw et al. 1993). First, each enzyme is designated by the reaction they carry out: AAC for acetylation, APH for phosphorylation, and ANT for adenylation. This is followed by the regiospecificity of the group transferred, designated in parentheses. Next follows a Roman numeral, which specifies the unique aminoglycoside substrate profile. A final lower case letter identifies the distinct genes which confer identical resistance phenotypes.

2.3

Aminoglycoside Phosphotransferases

Although many aminoglycoside-modifying enzymes are capable of conferring a resistant phenotype, generally only APHs yield high levels of resistance (Vakulenko and Mobashery 2003). APHs constitute the second largest group of aminoglycoside-modifying enzymes carrying out the phosphorylation of specific hydroxyl groups of aminoglycosides using ATP as a cofactor. Over 20 distinct aminoglycoside kinases have been identified, and they show significant sequence similarity (Nurizzo et al. 2003). APHs are classified into seven types (Table 1) (Shaw et al. 1993) and among them, APH(3') enzymes make up the largest group, comprising seven sub-classes [APH(3')-I to

Enzyme	Profile	Reference(s)
APH(3')-Ia	Kanamycin, neomycin, lividomycin, paromomycin, ribostamycin	Oka et al. 1981
APH(3')-IIa	Kanamycin, neomycin, butirosin, paromomycin, ribostamycin	Beck et al. 1982
APH(3′)-IIIa	Kanamycin, amikacin, isepamicin, gentamicin B, butirosin, lividomycin, paromomycin, ribostamycin,	Gray and Fitch 1983; Trieu-Cuot and Courvalin 1983
APH(2")-Ia (bifunctional enzyme)	Kanamycin, amikacin, tobramycin, dibekacin, gentamicin, isepamicin, sisomicin, netilmicin, neomycin, butirosin, lividomycin, paromomycin, ribostamycin, neamine	Daigle et al. 1999a; Ferretti et al. 1986

Table 2 Substrate profiles of selected aminoglycoside-modifying enzymes

APH(3')-VII]. They have been isolated from both gram-positive and gramnegative bacteria, including aminoglycoside-producing organisms (Shaw et al. 1993).

APH(3')-IIIa is the best-studied aminoglycoside kinase. It was originally isolated from Streptococcus faecalis (Trieu-Cuot and Courvalin 1983) and Staphylococcus aureus (Gray and Fitch 1983). Subsequently, the gene was detected in Campylobacter coli, which became a precedent for the transfer of antibiotic resistance gene transfer between gram-positive and gram-negative bacteria (Papadopoulou and Courvalin 1988; Taylor et al. 1988). The aph(3')-IIIa gene from Enterococcus faecalis has been cloned and the protein expressed and purified from Escherichia coli (McKay et al. 1994a). APH(3')-IIIa has one of the broadest substrate ranges among all single-function aminoglycoside-modifying enzymes (Table 2). As its name implies, APH(3')-IIIa catalyses the transfer of a phosphate group to the 3'-hydroxyl of the antibiotic substrate. Interestingly, it is also able to transfer a phosphate group from ATP to the 5" hydroxyl of neomycin-type aminoglycosides (McKay et al. 1994a; Thompson et al. 1996b). Hence, aminoglycosides which lack the 3'-hydroxyl, such as lividomycin, are also substrates for APH(3')-IIIa, since they can be modified at the 5"-position. In this sense, it should be named more appropriately as APH(3')(5'')-IIIa. The enzyme is 264 amino acid residues in size, with a molecular mass of 31,000 Da. The phosphorylation reaction catalysed by APH(3')-IIIa has been shown to follow a Theorell-Chance mechanism where ATP binds to the enzyme first, followed by the aminoglycoside; phosphorylated aminoglycoside is then quickly released, followed by the rate-limiting step-the release of ADP. Evidence suggests that the phosphoryl transfer occurs through direct attack of the 3'-hydroxyl group of the aminoglycoside on the γ -phosphate of ATP (Thompson et al. 1996a). APH(3')-IIIa is the first aminoglycoside kinase to be structurally characterized. The three-dimensional atomic structure of the enzyme complexed with

ADP was determined to 2.2 Å by X-ray crystallography (Hon et al. 1997) (see Sect. 3). Subsequently, structures of APH(3')-IIIa in the apoenzyme form (i.e. no substrates), in complex with the non-hydrolyzable ATP analogue adenosine $5'-(\beta,\gamma-\text{imino})$ triphosphate, AMPPNP (Burk et al. 2001), as well as APH(3')-IIIa bound with ADP and kanamycin A or neomycin B (Fong and Berghuis 2002), have been determined.

Two other APH(3') enzymes, APH(3')-Ia and APH(3')-IIa, have also been extensively characterized. The amino acid sequences of APH(3')-Ia, APH(3')-IIa and APH(3')-IIIa enzymes share approximately 30% identity. APH(3')-Ia is the most commonly disseminated APH in gram-negative bacteria. The aph(3')-Ia gene was discovered on transposon Tn903 in *E. coli* (Oka et al. 1981), encoding an enzyme that is able to phosphorylate a range of clinically useful aminoglycoside antibiotics (Table 2) (Shaw et al. 1993). Steady-state kinetic studies of APH(3')-Ia indicate that the phosphorylation reaction occurs via a rapid equilibrium random mechanism and, like APH(3')-IIIa, the phosphoryl group is believed to be transferred in a direct displacement manner (Siregar et al. 1995).

Although APH(3')-IIa is rarely found in clinical isolates, it is still a wellknown APH. The aph(3')-IIa gene, encoded on transposon Tn5 (Beck et al. 1982) is widely used in molecular biology as a selectable marker in both eukaryotic and prokaryotic studies. APH(3')-Ia and APH(3')-IIa have almost identical substrate spectra (Table 2). Recently, the crystal structure of APH(3')-IIa complexed with kanamycin has been solved (Nurizzo et al. 2003). It is essentially identical to the structure of APH(3')-IIIa with root mean square (rms) deviation for C α atoms of about 1.7 Å.

Another important resistance factor in gram-positive pathogens is APH(2")-Ia. The gene aph(2")-Ia is downstream of aac(6')-Ie, and they are conjointly expressed as one enzyme with two reaction centres. This bifunctional enzyme is capable of inactivating essentially all clinically available aminoglycosides containing a 2-deoxystreptamine ring (Ferretti et al. 1986; Leclercq et al. 1992) (Table 2) and is most prevalent in methicillin-resistant S. aureus (MRSA) isolates in many parts of the world (Schmitz et al. 1999; Udo and Dashti 2000; Ida et al. 2001). The regiospecificity of AAC(6')-Ie-APH(2")-Ia is not very stringent. In addition to acetylating the amino group at the 6'-position, the AAC domain can also acetylate the 6'-hydroxyl of lividomycin and paromomycin. Whereas APH(2") denotes the transfer of a phosphate group to the 2"-hydroxyl of the drug, phosphorylation at the 3'- and 3"-positions of most 4,5-disubstituted aminoglycosides, and the 5"-position of lividomycin, has also been observed (Daigle et al. 1999a). The phosphorylation reaction of APH(2")-Ia proceeds through a random rapid equilibrium mechanism (Martel et al. 1983). The amino acid sequence of APH(2'')-Ia is about 15% identical to that of APH(3')-IIIa.

3 3'-Aminoglycoside O-Phosphotransferase-Illa Structure and Similarity to Eukaryotic Protein Kinases

APH(3')-IIIa from enterococci and staphylococci is perhaps the best understood of the aminoglycoside-modifying enzymes. Experiments have revealed much about the kinetic properties, mechanism and structure of this enzyme. As a result, APH(3')-IIIa has become a useful model for the study of aminoglycoside kinases.

3.1 Tertiary Structure

The APH(3')-IIIa enzyme is composed of two lobes, a 94-residue N-terminal lobe and a larger 157-residue C-terminal lobe. The two lobes are joined by a 12-residue linker region containing a short β -strand and α -helix. The N-terminal lobe consists of a five-stranded antiparallel β -sheet, with an α -helix located between two β -strands and a short 3_{10} helix preceding the first β -strand. The architecture of the N-terminal lobe of APH(3')-IIIa is similar to that observed in eukaryotic protein kinases (ePKs) such as the catalytic subunits of cyclic AMP (cAMP)-dependant protein kinase (cAPK), casein kinase (CsK) and phosphorylase kinase (PhK) (Owen et al. 1995; Xu et al. 1995; Knighton et al. 1991). For structurally conserved main-chain atoms in the N-terminal lobe, the rms deviation in atomic position is only 1.8 Å between APH(3')-IIIa and cAPK. This is remarkable, given the fact that the sequence identity between the two enzymes is less than 6% for these residues.

The 12-residue linker that connects the N- and C-terminal lobes of APH(3')-IIIa is also structurally similar to the equivalent region in the structures of protein kinases such as cAPK. The linker consists of a short α -helix flanked by sections of random coil. The C-terminal lobe of APH(3')-IIIa can be divided into three sections: (1) a central core composed of two α -helices and a long hairpin-shaped loop incorporating two short sections of antiparallel β -sheet, (2) an insert region composed of two α -helices.

There are four areas in which APH(3')-IIIa differs significantly from ePKs such as cAPK (Fig. 2). First, in APH(3')-IIIa, the loop between β -strands 1 and 2 contains a one-residue insertion and adopts a different conformation than that observed in ePKs (*red*). While this loop is the location of the conserved GXGXXG motif in protein kinases, the motif is not observed in APH(3')-IIIa. Second, as a consequence of differences in the C-terminal lobes of APH(3')-IIIa and the protein kinases, the location of the α -helix in the linker region is shifted in APH(3')-IIIa with respect to where it is usually located in the protein kinases (*yellow*). Third, APH(3')-IIIa has a 60-residue insert in its C-terminal lobe (*blue*). Consisting of two α -helices and a 19-res-

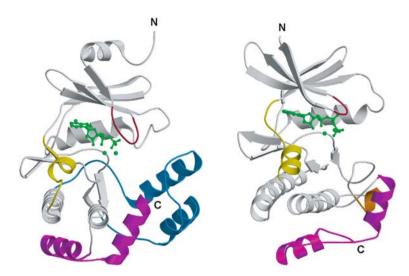


Fig. 2 A comparison of the structures of APH(3')-IIIa (*left*) and cAPK (*right*) highlighting, in colour, the areas with significant differences. These areas include: (1) the loop between strands β 1 and β 2 (*red*), (2) the linker region between the N- and C-terminal domains (*yellow*), (3) a 60-residue insert in APH(3')-IIIa (*blue*), (4) a smaller insertion found in ePK's (*orange*) and (5) the last two α -helices of the C-terminal domain (*magenta*). The ATP cofactor and its associated magnesium ions are depicted as green balls-and-sticks

idue loop, this insertion occupies the space of a smaller section of polypeptide found in ePKs (*orange*). The latter has a distinctly different conformation from that seen in APH(3')-IIIa and has been identified as important in substrate selectivity and specificity (Taylor et al. 1992; Madhusudan et al. 1994). This section of the ePK sequence also corresponds to the activation segment in some protein kinases, containing sites of phosphorylation that switch the enzyme from an inactive to an active conformation (Johnson et al. 1996; Yamaguchi and Hendrickson 1996). Lastly, the positions of the two α -helices at the end of the C-terminal lobes are significantly different in the APH(3')-IIIa and ePK structures (*magenta*).

3.2 ATP-Binding Site

The binding site for ATP and its associated magnesium ions in APH(3')-IIIa is located in the deep cleft between the N- and C-terminal lobes. Of the four absolutely conserved residues in both eukaryotic protein kinases and aminoglycoside phosphotransferases, three are located in the ATP-binding pocket where they interact with the cofactor and its associated magnesium ions—Asp190, Asn195 and Asp208 (Fig. 3). Two additional highly conserved residues, Lys44 and Glu60, are also found in the active site.

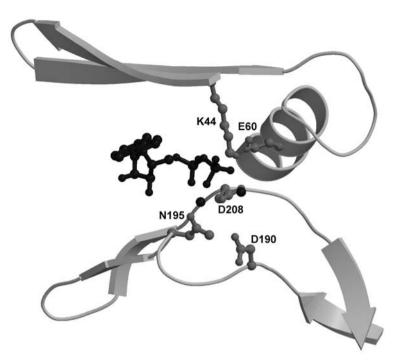


Fig. 3 The nucleotide-binding pocket of APH(3')-IIIa. The backbone is shown as *ribbon* and conserved residues are drawn as *balls-and-sticks*, both in *light grey*. ATP and its associated magnesium ions are drawn as *balls-and-sticks in black*

Lys44 is positioned over the binding site, interacting with the α - and β -phosphates of the cofactor. Evidence from mutagenesis studies suggests that Lys44 influences the $K_{\rm m}$ for ATP and thus makes an important contribution to ATP binding (Hon et al. 1997). The analogous lysine residue in the protein kinase family is also positioned to interact with the α - and β -phosphates of ATP. Glu60 is positioned in such a way that it hydrogen bonds to the side chain of Lys44, orienting it so that it interacts with the α - and β -phosphates of ATP.

In protein kinases, the residue corresponding to Asp190 has been proposed as a general base assisting in substrate deprotonation (Madhusudan et al. 1994). Although mutagenesis of Asp190 in APH(3')-IIIa results in drastically lower activity, supporting a role for this residue in catalysis, its role in catalysis has not been definitively identified (Hon et al. 1997; Zhou and Adams 1997). The function of Asp190 may be limited to positioning of the substrate hydroxyl group during phosphoryl transfer (Boehr et al. 2001b).

Mutagenesis studies of Asn195 indicated that this residue was important for ATP binding. Since Asn195 interacts with ATP via a magnesium ion, it has been suggested that the decrease in ATP affinity is the result of a nonoptimally coordinated metal ion (Boehr et al. 2001b). Asp208 is a ligand of both active site metal ions, and an Asp208Ala mutation results in a protein without detectable aminoglycoside kinase activity (Boehr et al. 2001b). Thus, Asp208 appears to be critical for catalysis in APH(3')-IIIa, facilitating the generation or stabilization of the transition state.

3.3 Aminoglycoside-Binding Site

The three-dimensional atomic structures of APH(3')-IIIa in complex with ADP and either the 4,6-disubstituted aminoglycoside kanamycin A or the 4,5-disubstituted aminoglycoside neomycin B have recently been determined (Fong and Berghuis 2002). Comparison of these structures with those of the apo, ADP and AMPPNP complexes of APH(3')-IIIa shows that most of the enzyme is rather rigid, with no evidence of gross domain movements associated with aminoglycoside binding. The most significant conformational change observed is a shift in the C-terminal insertion known as the aminoglycoside-binding loop. The loop folds over towards the antibiotic

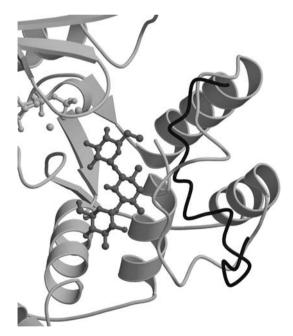


Fig. 4 An illustration of the APH(3')-IIIa aminoglycoside-binding pocket showing the conformational changes that occur upon aminoglycoside binding. The backbone of the kanamycin ternary structure of APH(3')-IIIa is shown as *ribbon* and the nucleotide cofactor and magnesium ions are drawn in *balls-and-sticks*, both in *light grey*. Kanamycin is shown as *balls-and-sticks in grey*. The antibiotic binding loop of the APH(3')-IIIa-ADP complex without bound aminoglycoside has been overlaid and coloured *black*. The movement of this loop is the largest conformational difference between the ternary and binary complexes

substrate, interacts with the aminoglycoside and completes the binding pocket (Fig. 4). The majority of the residues that line the aminoglycosidebinding pocket are acidic, and consequently the pocket is negatively charged. Thus, the binding pocket complements the aminoglycoside substrates, which are invariably positively charged.

The aminoglycoside-binding site of APH(3')-IIIa can be considered to be composed of three distinct binding subsites. The first of these, subsite A, interacts with the central 2-deoxystreptamine ring of the aminoglycoside and the hexose substituted at the 4-position of this central ring (the prime ring). The second subsite (subsite B) interacts with groups substituted at the 6-position of the 2-deoxystreptamine ring (the double prime ring). Consequently, this subsite is involved in binding only those substrates that have this type of modification, i.e. 4,6-disubstituted aminoglycosides. In contrast, the third subsite (subsite C) interacts with groups substituted at the 5-position of the central ring of 4,5-disubstituted aminoglycoside substrates.

Most of the hydrogen bond interactions observed between the aminoglycoside substrate and the APH(3')-IIIa enzyme are via subsite A. The central 2-deoxystrpetamine and prime rings that interact here are common to most aminoglycoside substrates and have been found to be the minimum essential components for antibacterial activity. There are fewer interactions between the aminoglycoside and subsites B and C. There is more variability in the parts of the aminoglycosides that interact here, and only functional groups that are conserved interact with the enzyme. While both subsites B and C are involved in fewer interactions than is subsite A, they differ significantly in size. This may be the consequence of the fact that the aminoglycoside substituents at the 6-position (with which subsite B interacts) are single hexose rings, whereas substitutions at the 5-position (with which subsite C interacts) can be one, two or three sugar rings.

4 Circumventing Aminoglycoside Inhibition by 3'-Aminoglycoside O-Phosphotransferase

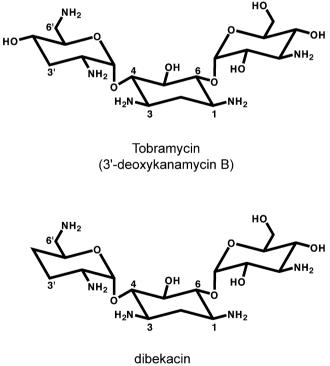
Two main principles are followed in the search of ways to circumvent inactivation of aminoglycosides by modifying enzymes. The first involves abolishing the resistance mechanism such that antibacterial activity can be restored. The second approach requires the development of compounds that are effective inhibitors of bacterial protein translation and can also evade resistant mechanisms. Most of these studies have been done with the phosphotransferase class of enzyme due to the amount of information available. Many compounds were developed based on the binding properties of the aminoglycosides, as well as on the kinetic mechanism of the aminoglycoside-modifying enzymes. Additionally, structural information has added much insight in the development of aminoglycoside derivatives and inhibitors targeted at the cofactor binding site or the whole binding cleft.

4.1 Targeting the Aminoglycosides and Their Binding Pocket

Most studies of strategies to circumvent the effects of resistance factors have focussed on aminoglycoside binding. The advantage of such an approach is that since the compound would necessarily mimic features of an aminoglycoside, it could be a universal inhibitor for all three classes of aminoglycoside-modifying enzymes.

4.1.1 Removing the Target Functional Group

There have been some successes in modifying existing aminoglycoside antibiotics to generate new compounds that bind, but are not inactivated



(3',4'-dideoxykanamycin B)

Fig. 5 Structures of the aminoglycosides tobramycin (*top*) and dibekacin (*bottom*). Both molecules lack a 3'-hydroxyl group, making them resistant to modification by APH(3')-IIIa

by, resistance enzymes. For example, tobramycin (3'-deoxykanamycin B) (Umezawa et al. 1971b) and dibekacin (3',4'-dideoxykanamycin B) (Umezawa et al. 1971a), both lacking the 3'-hydroxyl, are competitive inhibitors for APH(3')s (McKay and Wright 1995) (Fig. 5). Both molecules evade modification by the APH(3') enzymes. However, they can be deactivated by other classes of aminoglycoside-modifying enzymes.

4.1.2 Reducing the Binding Affinity

Another approach involves modifying the antibiotic in such a way that it can no longer bind to the aminoglycoside-modifying enzymes. This strategy is based on the observation that the naturally occurring butirosins, which possess a 4-amino-2-hydroxybutyryl (AHB) group on the amine at the 1-position of the 2-deoxystreptamine ring, are resistant to many inactivating enzymes, yet retain their bactericidal properties (Tsukiura et al. 1973) (Fig. 6a). It is thought that the AHB and other side chains at the 1-amino position hinder binding to the aminoglycoside-modifying enzyme (Kondo and Hotta 1999). This observation led to the development of second generation semisynthetic aminoglycoside antibiotics such as amikacin (Fig. 6b) and arbekacin (kanamycin A and dibekacin derivated at the N1 by an AHB group, respectively) (Kawaguchi et al. 1972; Kondo et al. 1973a,b; Holm et al. 1983), isepamicin (Fig. 6c) (gentamicin B substituted with a 4-amino-2-hydroxypropionyl at N1) (Nagabhushan et al. 1978), as well as netilmicin (sisomicin with ethyl group introduced at N1) (Wright 1976). They have been shown to be clinically useful, especially arbekacin, which is effective against MRSA infections (Kondo and Hotta 1999) and whose antibiotic activity is unaffected by 2'- and 3"-acetylation (Hotta et al. 1996, 1998). Unfortunately, some level of resistance has been noted, chiefly as a consequence of inactivation by the bifunctional enzyme AAC(6')-Ie-APH(2")-Ia (Kondo et al. 1993b; Fujimura et al. 1998; Fujimura et al. 2000). Subsequently, two derivatives of arbekacin, 2"-amino-2"-deoxyarbekacin and 2"-amino-5,2"-dideoxy-5-epiaminoarbekacin (Kondo et al. 1993a, 1994), have been developed and shown to be active in vivo, yet less toxic to mammals than their parent compound (Inouye et al. 1996).

Another scheme for diminishing the binding affinity of aminoglycosides for APHs is by minimizing the electrostatic interactions between the aminoglycoside and the resistance enzyme. This is achieved through the deletion of amino or hydroxyl groups at important positions on neamine and kanamycin B (Roestamadji et al. 1995a; McKay et al. 1996). These modified drugs retain their antibacterial activity but have a significantly reduced rate of phosphorylation and affinity for APH(3')-Ia and APH(3')-IIa, probably due to the removal of specific ionic and hydrogen bond interactions between the

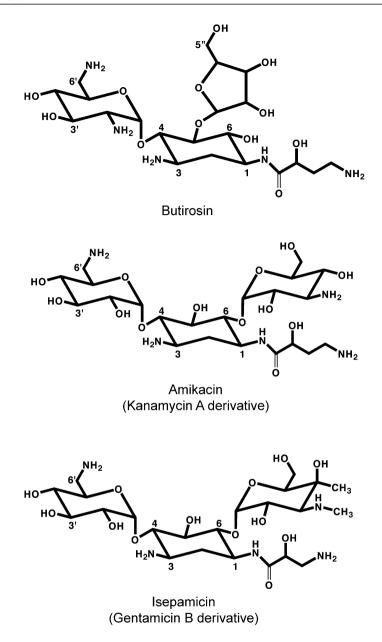


Fig. 6 Three aminoglycosides with substitutions at the 1-amino group of the central 2-deoxystreptamine ring. Butirosin is naturally occurring, while amikacin and isepamicin are semisynthetic aminoglycosides. The 1-amino substitutions are believed to hinder binding to aminoglycoside-modifying enzymes, making these compounds resistant to inactivation

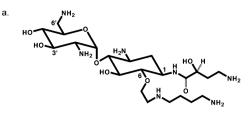
substrate and the enzyme. However, the affinity of these analogues for APH(3')-IIIa is only moderately affected.

4.1.3 Neamine Derivatives

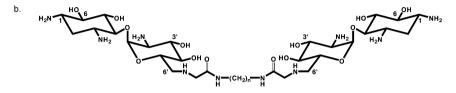
Neamine is a poor antibiotic and is not clinically useful. However, it serves as an invaluable template for the design of new antibiotics. It has been shown that neamine is the minimal structural motif required for binding to the A-site of 16S subunit of ribosomal RNA (Fourmy et al. 1996, 1998). Hence, it is sensible, in designing new aminoglycosides, to preserve the minimum structural motif required for RNA binding and antibiotic activity, but to deviate from typical aminoglycoside structures, in order to elude the various modifying enzymes.

Previous studies showed that the antibiotic activity can be retained when ring IV of neomycin B is substituted with a diaminoalkane group, even though the analogue binds to RNA with diminished specificity (Alper et al. 1998). Subsequently, Greenberg et al. synthesized derivatives of neamine by appending various poly amino, amino alcohol, or aromatic substitutions at the O5-position (Greenberg et al. 1999). The results showed that the compounds substituted with a diaminoalkyl group enhanced the binding to RNA while exhibiting antibiotic activity equivalent to neamine.

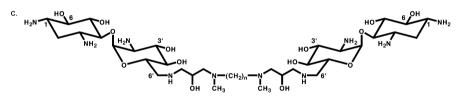
More recently, several neamine derivatives were synthesized based on the interactions observed in the nuclear magnetic resonance (NMR) solution structure of paromomycin bound to an A-site rRNA template, as well as extensive searches in the Cambridge Structural Database and the National Cancer Institute 3-D Database (Haddad et al. 2002). These compounds are composed of a neamine core, with an AHB group or its analogue at the N1position of the 2-deoxystreptamine (as in butirosin and amikacin), plus an diaminoalkane group of various lengths at the O6-position Fig. 7a). An AHB group was selected as a substituent, since aminoglycosides such as butirosin and amikacin, which possess this structure at N1, have reduced affinity for aminoglycoside-modifying enzymes (see Sect. 4.1.2). The terminal aminecontaining aliphatic component was added in order to improve the interaction between the O6 and the phosphate backbone of the target rRNA. Many of the designed compounds were shown to be capable of binding to a fragment modelling the A-site of the E. coli rRNA, and demonstrated broad spectrum antibiotic activity that is much higher than their parent compound or equal to that of other commonly used aminoglycosides. Some of these designed antibiotics were shown to be poor substrates of APH(3')-Ia and AAC(6')-Ie-APH(2")-Ia, the bifunctional enzyme. The crystal structure of the A-site rRNA template in complex with a designer neamine derivative of high antibiotic activity has recently been reported (Russell et al. 2003). The structure showed that the binding mode of the designer compound is essentially identical to that of paromomycin (Carter et al. 2000). Comparison



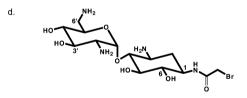
Neamine derivated at N1 and O6



Neamine dimers linked by amides



Neamine dimers linked by 1,2-hydroxyamines



A bromoacetylated neamine

Fig. 7a–d Four synthetic aminoglycosides based on the structure of neamine, the minimal structure required for binding to the bacterial ribosome. **a** A neamine derivative with AHB and diaminoalkane substitutions at the N1 and O6 positions, respectively. **b** Amine-linked neamine dimers. **c** 1,2-hydroxyamine-linked neamine dimers. **d** A bromoacetylated neamine

of neamine derivatives to the kanamycin A bound to APH(3')-IIIa (Fong and Berghuis 2002) also explains the basis of the designer molecules' ability to elude inactivation by aminoglycoside-modifying enzymes. The AHB moiety at the N1-position forms steric clashes with the antibiotic binding loop of

APH(3')-IIIa, impeding the formation of an active ternary complex (Russell et al. 2003).

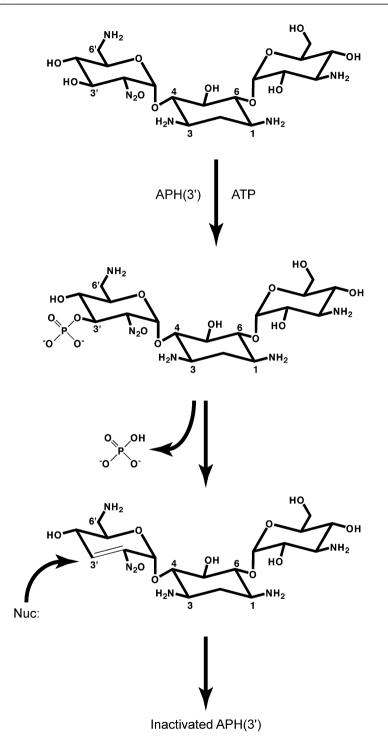
It has been shown that neamine binds to the A-site model of prokaryotic rRNA in a 2:1 ratio (Sucheck et al. 2000). A series of neamine dimers were constructed in order to identify bivalent aminoglycosides that would interact with the model target site of aminoglycoside in bacteria, and at the same time resist modification by aminoglycoside-modifying enzymes due to its dramatically altered and unusual structure. Two neamine molecules are joined by either amides or 1,2-hydroxyamine with methylene bridges of variable lengths (Fig. 7b,c). These compounds were found to possess antibiotic activity that is comparable or even superior to that of neamine. They are also potent competitive inhibitors of APH(2'') activity of the bifunctional enzyme AAC(6')-Ie-APH(2'')-Ia and are poor substrates for APH(3')-IIIa and the AAC(6')-Ie activity of the bifunctional enzyme.

Four derivatives of neamine have also been synthesized by regiospecifically appending a bromoacetyl group to the various amines of the antibiotic (Roestamadji and Mobashery 1998) (Fig. 7d). The affinity of the bromoacetylated compounds for APH(3')-IIa is significantly reduced. In the presence of ATP, the phosphorylation reaction would proceed but at an attenuated rate, whereas in the absence of ATP, the bromoacetylated neamines would inactivate APH(3')-IIa in a time-dependent and saturable manner. Moreover, the activity of the enzyme could not be recovered despite extensive dialysis, in an attempt to remove the modified neamine molecules. This observation suggests that the electrophilic bromoacetyl group could form covalent bonds with different nucleophilic residues in the active site. As a result, the modified neamine becomes irreversibly bound to the enzyme and prevents it from binding and inactivating aminoglycosides.

4.1.4 Mechanism-Based Inhibition

The first mechanism-based inhibitors of APH(3')s were described by Roestamadji et al. (1995b). The compounds are derivatives of neamine and kanamycin B, in which a nitro (NO₂) group replaces the amine at the 2'-position (Fig. 8). These suicide substrates are excellent substrates for APH(3')s but poor antibiotics. Upon phosphorylation by APH(3') enzymes at the 3'-hydroxyl, the phosphoryl group, being an excellent leaving group, is

Fig. 8 Proposed mechanism of aminoglycoside kinase inhibition by 2'-nitro aminoglycoside derivatives. Spontaneous loss of phosphate from a phospho-aminoglycoside yields an electrophilic nitroalkene. Trapping of a nucleophilic active site residue (*Nuc*) produces an inactivated enzyme



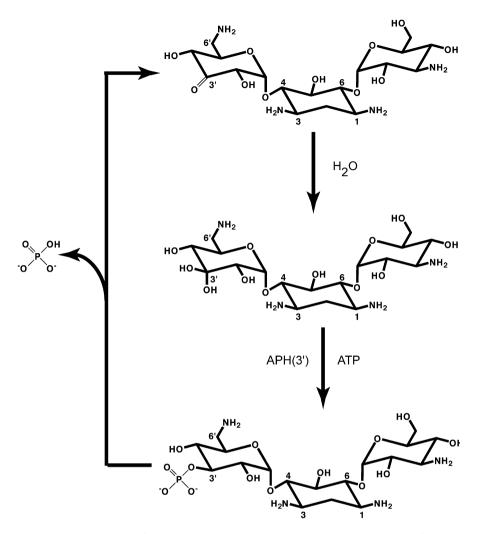


Fig. 9 Reaction cycle of 3'-oxo-kanamycin. Although a good substrate for APH(3') phosphotransferases, the phosphorylated product is unstable and is spontaneously dephosphorylated to regenerate the original compound

rapidly eliminated, generating an electrophilic nitroalkene. The reactive electrophilic intermediate can in turn capture an active-site nucleophilic amino acid side chain and form a covalent bond, irreversibly inactivating the enzymes.

A more recent derivative is 3'-oxo-kanamycin A, a self-regenerating aminoglycoside, in which the hydroxyl group at 3'-position is replaced by a ketone (Haddad et al. 1999) (Fig. 9). The hydrated variant of this compound is a good substrate for APH(3') enzymes. However, the phosphorylated prod-

uct is unstable and releases the inorganic phosphate in a spontaneous nonenzymatic way, regenerating the parent compound. The antibiotic is therefore not inactivated, making the resistance enzymes obsolete.

The molecular recognition of aminoglycosides in both the ribosomal Asite and all three classes of resistance enzymes is accomplished in analogous ways. In the case of APH(3') enzymes, this is illustrated by comparing the binding of paromomycin I to the 16S ribosomal RNA (Carter et al. 2000) and neomycin B to APH(3')-IIIa (Fong and Berghuis 2002). The crystal structure of the 30S ribosome in complex with different aminoglycosides (including paromomycin I) has been solved (Carter et al. 2000). Paromomycin I is a 4,5-disubstituted 2-deoxystreptamine aminoglycoside that can be inactivated by many APH(3') enzymes. The structure of paromomycin I closely resembles that of neomycin B; the only difference is in the functional group at the 6'-position, where paromomycin I has a hydroxyl and neomycin B an amino group. Comparison of the binding of neomycin B to APH(3')-IIIa and paromomycin I to the A-site of the bacterial ribosome reveals that the conformation of the aminoglycosides and the hydrogen bond network between the aminoglycoside and their respective targets are essentially identical. However, the two complexes differ considerably in their van der Waals interactions. The face of the aminoglycoside that makes most of the van der Waals interactions with APH(3')-IIIa is opposite to that which interacts with the bacterial ribosome (Fong and Berghuis 2002) (Fig. 10). The similarities in aminoglycoside binding to the two targets explain the effectiveness of APH(3')-IIIa as a resistance factor, but more importantly, the differences in binding mechanism can be exploited in the design strategies of inhibitors

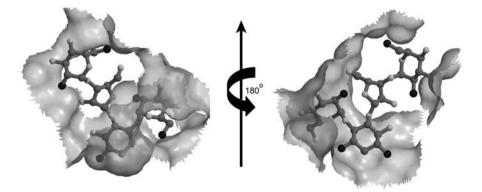


Fig. 10 The van der Waals surface of the aminoglycoside binding site of APH(3')-IIIa (*left*) and the bacterial ribosome (*right*). APH(3')-IIIa is shown with bound neomycin B, while the ribosome structure has bound paromomycin I. Atoms of the ball-and-stick illustrations of the aminoglycoside are colour coded as follows: oxygen, *light grey*; carbon, *grey*; nitrogen, *black*

and novel aminoglycoside variants. For example, the binding of aminoglycoside derivatives can be blocked by modifying the corresponding face with bulky chemical moieties (Fong and Berghuis 2002; Vicens and Westhof 2003).

4.2 Targeting the Nucleotide-Binding Pocket

Studies on aminoglycoside kinase inhibitors that target the nucleotide binding site were prompted by the elucidation of the three-dimensional structure of APH(3')-IIIa. As discussed above, the overall structure of APH(3')-IIIa is remarkably similar to that of eukaryotic protein kinases, especially in the ATP binding domain (see Sect. 3). This finding led to the survey of the effectiveness of a wide range of ePK inhibitors such as the indole carbazoles, the flavonoids, and the isoquinolinesulphonamides (which are all competitive inhibitors of ATP) against aminoglycoside kinases (Daigle et al. 1997). The basis of this study was that molecules that target the nucleotide-binding site would prevent the binding of ATP and thus disrupt the enzyme function. One limitation of this approach of inhibitor design is that only those aminoglycoside-modifying enzymes that use ATP cofactors would be inhibited. Of the three classes of ePK inhibitors tested, the isoquinolinesulphonamides (Fig. 11a) have been found to be good competitive inhibitors of ATP for both APH(3')-IIIa and the bifunctional enzyme, AAC(6')-Ie-APH(2")-Ia. Structural studies reveal that these inhibitors bind to the active site of protein kinases by forming a hydrogen bond between the nitrogen of the isoquinoline and the main chain amide hydrogen of the linker peptide that joins the N- and the C-terminal domains (Engh et al. 1996; Xu et al. 1996). An analogous hydrogen bond is also observed between the N1 of the purine ring in ADP or AMPPNP and the linker of APH(3')-IIIa. Therefore, it is inferred that isoquinolinesulphonamides would bind to APH(3')-IIIa in a similar fashion and thus inhibit the function of APH(3')-IIIa. Unfortunately, these compounds are only able to inhibit the resistance enzymes in vitro and cannot reverse antibiotic resistance in enterococcal strains that harbour either the aph(3')-IIIa or aac(6')-Ie-aph(2'')-Ia gene (Daigle et al. 1997).

Protein kinases play key roles in virtually every activity and signalling pathway involved in the development and maintenance of eukaryotic cells. Therefore, care must be taken in the design of inhibitors directed at the nucleotide-binding pocket of aminoglycoside kinases to avoid cross-reactivity. Structural information about the proteins becomes particularly valuable in this respect. By examining the structures of APHs and ePKs, it is possible to identify distinguishing features that might be utilized in the development of compounds that would selectively inhibit APHs without affecting host protein kinases (Boehr et al. 2002). For example, the crystal structure of APH(3')-IIIa showed that Tyr42 is located near the nucleotide binding site

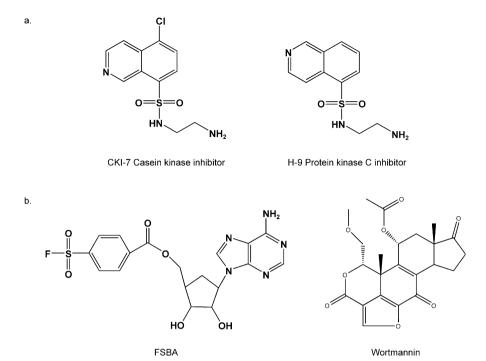


Fig. 11 a Structures of two isoquinolinesulphonamide inhibitors of protein kinases. **b.** Structures of FSBA (*left*) and wortmannin (*right*), protein kinase inhibitors that target the nucleotide-binding site

and that its aromatic ring side chain forms stacking interactions with the adenine moiety of ATP. This residue is highly conserved as either a tyrosine or phenylalanine among APH(3') enzymes, but it is predominantly an alanine in protein kinases (Burk et al. 2001). The orientation of the adenine ring in protein kinases and APH(3')-IIIa also differs by a rotation of about 40° due to the difference in electrostatic interactions in the nucleotide binding site (Burk et al. 2001; Burk and Berghuis 2002). It is possible that such a difference could be exploited to produce an inhibitor specific to APHs, eluding the problem of protein kinase inhibition by APH inhibitors.

Another complication to overcome in the development of broad spectrum antibiotics that target the nucleotide binding site are the differences in the active sites between various APHs. As described earlier in this section, APH(3')-IIIa and AAC(6')-Ie-APH(2")-Ia display definite selectivity towards one specific class of protein kinase inhibitor, implying that the nucleotide binding sites have critical differences (Boehr et al. 2001a). This issue was further illustrated by Wright et al. using 5'-[p-(fluorosulfonyl)benzoyl]adenosine (FSBA) and wortmannin (Fig. 11b) (Boehr et al. 2001a). Previous experiments showed that FSBA, a hydrophilic ATP analogue, inactivates

APH(3')-IIIa by covalently linking to invariant Lys44 in the nucleotide-binding site (McKay et al. 1994b). However, FSBA has no effect on the APH(2")-Ia portion of the bifunctional enzyme (Boehr et al. 2001a). Conversely, wortmannin, a potent inhibitor of phosphoinositol 3-kinase (Powis et al. 1994), was able to inhibit APH(2")-Ia but not APH(3')-IIIa. Phosphatidylinositol kinase, which shares a similar fold as aminoglycoside and protein kinases (Rao et al. 1998), can be inhibited by wortmannin, which covalently modifies Lys802 in the ATP-binding pocket (Wymann et al. 1996). Wortmannin inactivates APH(2")-Ia in the same manner by covalently binding to Lys52 [homologous to Lys44 in APH(3')-IIIa] (Boehr et al. 2001a).

4.3 Exploiting the Bridged Binding Site

The feasibility of designing inhibitors for resistance enzymes that target the binding sites of both the cofactor and the aminoglycoside is based on the enzyme mechanism of APH(3')s. Whereas APH(3')-Ia functions by a random equilibrium BiBi mechanism (Siregar et al. 1995), APH(3')-IIIa catalyses its reaction by the Theorell-Chance mechanism, a form of ordered BiBi mechanism (McKay and Wright 1995). Both mechanisms require that all substrates must be present in the active site prior to catalysis. Using this approach, tethered derivatives of adenosine and the aminoglycoside neamine have been synthesized (Liu et al. 2000). These bisubstrate analogues are made by covalently linking the 5'-hydroxyl of adenosine to the 3'-hydroxyl of neamine via methylene linkers of various lengths (Fig. 12). Appropriate linker lengths that very nearly span the distance between the ATP and the aminoglycoside binding sites should manifest themselves by showing strong inhibition of the resistance enzyme. When tested against the APH(3')-Ia and APH(3')-IIIa, compounds with linkers of 6-7 carbons in length were found to be the most potent competitive inhibitors of both ATP and kanamycin A.

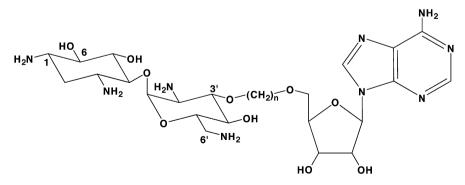


Fig. 12 A tethered derivative of adenosine and neamine that targets both the nucleotideand substrate-binding sites of aminoglycoside-modifying enzymes

These bisubstrate inhibitors must contain many elements required for binding both regions of the active site of aminoglycoside kinases. The specificity of the molecule is increased and the problem of cross-reactivity with host protein kinases can be circumvented. Nevertheless, the breadth of activity of these molecules is reduced, since they are able to bind only those resistance factors that utilize ATP. In addition, the requirement for sufficient specificity in both binding pockets means that compounds that are developed to meet these conditions are likely to be large. Such compounds are unlikely to be effective therapeutic agents, due to issues associated with membrane transport of large molecules (Burk and Berghuis 2002).

4.4

Alternative Strategies for Circumventing Antibiotic Resistances

In addition to the investigation of the inhibition of APHs using ePK inhibitors that target ATP binding, the discovery of the structural similarities between aminoglycoside and protein kinases also led to the examination of the ability of APHs to phosphorylate protein kinase substrates (Daigle et al. 1999b). APH(3')-IIIa and APH(2")-Ia (of the bifunctional enzyme) were shown to be capable of phosphorylating peptide substrates, but at a much slower rate than the phosphorylation of aminoglycosides. The binding modes of peptide substrates were examined by modelling studies using the structure of APH(3')-IIa. The crystal structure of APH(3')-IIa in complex with kanamycin A is the newest addition to the array of structural information on aminoglycoside-modifying enzymes (Nurizzo et al. 2003). The cAMP kinase inhibitor PKI in the conformation observed in the crystal structure of cAMP-dependent protein kinase (Zheng et al. 1993) was modelled into the active site of APH(3')-IIa (Smith and Baker 2002). The peptide can be readily accommodated in the binding site of APH(3')-IIa with very few steric hindrances detected between the inhibitor and the resistance enzyme.

The possibility of using cationic peptides as starting molecules for the development of broad-spectrum inhibitors of resistance enzyme activities was therefore examined (Boehr et al. 2003). Due to the large number of negatively charged residues in the binding pockets of aminoglycoside-modifying enzymes, positively charged peptides are the preferred substrates. Both APH(3')-IIIa and APH(2")-Ia were inhibited by protegrin, indolicidin and its analogue, CP10A, in a non-competitive manner with both ATP and kanamycin. The inhibition patterns demonstrate that the peptides bind to both the free enzyme and to enzyme-substrate complexes. Together, these results suggest that the peptide inhibitors have multiple binding modes and may span both the ATP and aminoglycoside binding sites. Furthermore, the modelling study of cAMP kinase PKI and APH(3')-IIa corroborated the inhibition kinetics experiments that showed that PKI fully occupied the aminoglycoside binding site as well as a large portion of the putative ATP binding site (Smith and Baker 2002). Although some antimicrobial peptides are able to penetrate the cytoplasm of the bacterium, many are known to act on the cytoplasmic membrane (Wu et al. 1999). Unfortunately, none of the peptides that inhibited resistance enzymes in vitro displayed synergistic antimicrobial properties with aminoglycosides in organisms harbouring resistance genes (Boehr et al. 2003).

5 Conclusions

Despite the tremendous success of antibiotics over the past 50 years, infectious diseases remain a serious problem for public health due to the prevalence of antibiotic-resistant pathogens. Aminoglycoside resistance is no longer restricted to the hospital environment, but now represents a problem for communities in both developing and developed worlds. To date, there is no known inhibitor of aminoglycoside-modifying enzymes that can be used in combination with existing aminoglycosides in a clinical setting. Ideally, a single inhibitor would be designed that would block the function of all the aminoglycoside-modifying enzymes. However, the design of a molecule that would bind effectively to binding sites of all three classes of enzymes would be extremely difficult. Although the negatively charged aminoglycosidebinding sites are a common feature in all three classes of resistant enzymes, differing ranges of substrate specificity and regiospecificity give rise to distinct features in the various classes of enzymes. However, it is plausible to develop a compound which would effectively block the activity of one family of enzymes, despite subtle differences between enzymes within each family. The design of new antibacterial agents that bind to the A-site of the bacterial ribosome is also equally challenging. A newly designed molecule has to fulfill an array of prerequisites before it can be considered as a drug candidate. It will have to be taken up into the bacterial cell, bind to the bacterial ribosome, interfere with protein translation and be a poor substrate for resistance enzymes. Given the findings on aminoglycoside inhibition by APHs described here and as more mechanistic and structural information on aminoglycoside-modifying enzymes becomes available, it is possible that the restoration of current aminoglycosides as well as the development of new aminoglycoside and nucleotide derivatives with antibacterial activity may indeed be possible in the future.

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Part III Pharmacological Potential and Inhibitors of Individual Classes of Protein Phosphatases

Protein Tyrosine Phosphatases as Therapeutic Targets

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Abstract The protein tyrosine phosphatases (PTPs) are a family of regulatory enzymes that are critical for a wide variety of cellular functions. During the last decade, the interest and findings on the role of PTPs in signal transduction have increased tremendously. Initially, these enzymes were viewed as negative regulators of signaling cascades triggered by protein tyrosine kinases (PTKs). It is now well established that several members of the PTP family are also positive regulators of several signaling pathways. Compelling evidence from biochemical, cell biology, and gene-targeting studies has implicated PTPs in multiple processes that include metabolism, development, immunity, and cancer. Indeed, mutations in several PTP genes are associated with human disorders. Moreover, many

pathological organisms have acquired specific PTPs or target host cell PTPs to increase virulence and promote their own propagation. Taken together, these findings identified specific PTPs as significant targets for the therapeutic intervention in human diseases. For instance, the discovery that PTP1B is a negative regulator of insulin signaling revealed that this molecule is an attractive target for the treatment of insulin-resistant diseased states like type 2 diabetes mellitus and obesity. At the other end of the spectrum, the phosphatases of the CDC25 subfamily, which are key regulators of the cell cycle, are currently targeted for the treatment of cancers. These are just two examples that are discussed in this review. Although, we are still in the infancy in understanding PTP function, several of these enzymes are currently intensely investigated for their potential therapeutic values.

Keywords Protein tyrosine phosphatase · Tyrosine phosphorylation · Signal transduction · Microbial phosphatase · Therapeutic targets

1 Introduction

1.1 Background

Reversible phosphorylation on tyrosine residues represents an important means by which cells regulate signal transduction. Tyrosine phosphorylation is known to alter protein conformation, modulate enzymatic activity, and also create binding sites for the recruitment of signaling proteins involved in the regulation of many basic biological processes such as cell proliferation, differentiation, migration, apoptosis, and embryogenesis (Blume-Jensen and Hunter 2001). The control of intracellular tyrosine phosphorylation levels depends on the coordinated action of two opposing enzymes, the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPs). To date, most studies have focused on PTKs, and, subsequently, much remains to be discovered concerning the physiological functions of PTPs. Nevertheless, several enzymes within this family have recently received much attention as attractive targets for the treatment of several diseases, including diabetes and cancer.

1.2 PTPs and Impact on Public Health

In the last 5 years, the PTP field has rapidly gained special interest with particular emphasis on PTP1B. Gene targeting studies in mice strongly supported that PTP1B is involved in the insulin resistance associated with type 2 diabetes mellitus (Elchebly et al. 1999a; Klaman et al. 2000a). This disease and associated disorders are becoming a serious threat to human health with a global figure of about 150 million people affected. Moreover, mutations in genes coding for different members of the PTP family have been associated with various congenital diseases (Minassian et al. 1998; Serratosa et al. 1999; Digilio et al. 2002; Legius et al. 2002; Tartaglia et al. 2002). Overexpression of several PTPs has also been observed in several human cancers, including breast and colon cancers (Tabiti et al. 1995; Lee et al. 2000; Pestell et al. 2000; Saha et al. 2001). These observations together indicate that PTPs are key factors of many different diseases with a wide impact on public health. PTPs are also critical factors for several pathogens that cause a large spectrum of diseases worldwide, amongst which typhoid fever is still responsible for 600,000 deaths annually (Pang et al. 1998) and Helicobacter pylori, which is responsible for gastric ulcer and malignancy, infects about half of the world population (Rothenbacher and Brenner 2003). Thus, selected PTPs may also represent novel targets against particular infectious diseases. In this review, we will describe the recent findings demonstrating the implication of PTPs in human diseases and health care. We will also discuss the therapeutic potential of targeting various members of the PTP family.

2 Review

2.1 PTP Family

Members of the PTP superfamily of structurally related enzymes are defined by a highly conserved catalytic domain. Within this 250 amino acid region, the active site signature sequence [HCXXGXXR] contains the invariant cysteine residue that is critical for PTP activity (Tonks and Neel 2001). Sequence data from the human genome and recent estimates from the literature predict the existence of 117 members for the PTP family (see the Gene Ontology Consortium at http://www.geneontology.org/ and http:// science.novonordisk.com/ptp/). Based on their sequences, structures, and functions we divided the PTP family into three categories: (1) the low molecular weight PTPs (LMW PTPs), (2) the dual-specific phosphatase (DSP), and (3) the phosphotyrosine-specific enzymes (classical PTPs).

LMW PTPs represent a small group of 18-kDa enzymes with broad tissue expression (Raugei et al. 2002). Overexpression of LMW PTPs in cells has been shown to dephosphorylate the platelet-derived growth factor receptor (PDGFR) (Chiarugi et al. 1995), p190RhoGTPase-activating protein (p190RhoGAP) (Chiarugi et al. 2000), as well as the epinephrineA2 (EphA2) kinase (Kikawa et al. 2002). These studies link LMW PTPs to both growth factor and adhesion signaling, suggesting that they may be important for many cellular processes, including mitogenesis and transformation. Recently, LMW PTP was also proposed to play a positive role in T cell receptor signal-

ing similar to the one proposed for CD45 (Bottini et al. 2002). However, the physiological functions of these enzymes remain unclear.

DSPs have the ability to dephosphorylate both tyrosine and serine/threonine residues. The most prominent members include the Vaccinia virus phosphatase-related enzymes, cell division cycle 25 (CDC25), and the mitogen-activated protein kinase (MAPK) phosphatases (MKPs). The best-characterized functions of these examples involve the regulation of the MAPK pathway and cell cycle progression (Keyse 2000; Lyon et al. 2002). Another interesting group of DSPs include the PTEN (phosphatase and tensin homolog deleted on chromosome 10) and myotubularin phosphatases (Maehama et al. 2001). Although the sequence of their catalytic domains is similar to other DSPs, both PTEN and myotubularin act primarily as phosphoinositide phosphatases. In contrast, their activity on protein substrates remains in doubt. Nevertheless, the importance of these two enzymes is highlighted by the presence of mutations that are responsible for severe human diseases, including cancer and muscular development.

The classical PTPs—the major group of the PTP family—comprise two groups, the intracellular and transmembrane (receptor type) enzymes, from which 17 subtypes were derived based upon their sequence similarity among catalytic PTP domains (Andersen et al. 2001). The receptor type PTPs generally contain an extra-cellular segment with putative ligand-binding domains, a single transmembrane region and one or two cytoplasmic catalytic domains, where the first domain contains most if not all catalytic activity. The intracellular PTPs have a single catalytic domain and varying amino- or carboxy-terminal extensions containing protein-binding domains that are believed to have targeting or regulatory functions (Tonks and Neel 2001).

Despite the large heterogeneity in all the PTP subgroups, the crystal structure of several members (mostly catalytic domains) revealed that they all display a similar core folding feature (Barford 1999; Andersen et al. 2001). Thus, this common active site structure suggests that these enzymes use a similar mechanism for catalysis (Zhang 1998; Li and Dixon 2000; Kolmodin and Aqvist 2001).

2.2 Enzyme/Substrate Specificity

The above observations raise questions as to how enzyme/substrate specificity can be achieved despite common active site structure. Although binding affinity is primarily dependent on the catalytic site, the specificity is mainly achieved by a substrate-induced conformational change through specific interactions involving contacts outside the catalytic site that favor catalysis (Barford 2001; Zhang et al. 2002). In the case of the classical PTPs the situation is not as clear and was recently reviewed (Ostman and Bohmer 2001; Tonks and Neel 2001). A likely model is that, in vivo, individual PTPs act against a limited number of cellular targets and that a given cellular target is probably regulated by more than one PTP. However, it appears that each PTP performs a unique interaction with a substrate, indicating that intrinsic catalytic domain specificity exists (Tonks and Neel 2001). Recent studies on the DSP Cdc14 elegantly demonstrate how this can occur (Gray et al. 2003).

A variety of protein interacting domains have been described for PTPs, including Src homology 2 (SH2) domains, proline-rich motifs (interacting with SH3 domains), PDZ domains, and band 4.1 homology domains (Li and Dixon 2000; Ostman and Bohmer 2001; Tonks and Neel 2001; Raugei et al. 2002). Targeting signals to the nucleus or the endoplasmic reticulum have also been identified in some PTPs. Post-translational isoprenylation of members of the PTP4A subfamily was suggested to help direct these molecules to the plasma membrane (Cates et al. 1996) Therefore, substrate selectivity is dependent to some extent on the protein interacting motifs and targeting signals contained in each intracellular PTP. Following these lines, it is evident that several mechanisms such as structural properties, subcellular localization, associated proteins, and others, concur to restrict the PTP members to their corresponding substrates and thereby specifying their physiological roles.

2.3 Regulation of PTP Action

Temporal activation of PTPs is another important factor in the regulation of PTP action. Activation can be regulated at the transcriptional level, or by post-translational mechanisms such as phosphorylation on serine/threonine residues (Draetta and Eckstein 1997; Digilio et al. 2002; Zheng et al. 2002), or on tyrosine residues (Ostman and Bohmer 2001; Raugei et al. 2002). Furthermore, enzymatic activity can be inhibited rapidly and reversibly by oxidation of the essential catalytic cysteine residue (Mahadev et al. 2001; Meng et al. 2002; Raugei et al. 2002). Intramolecular interactions, such as disulfide bonds (Caselli et al. 1994; Caselli et al. 1998) and blockade of the catalytic site by SH2 domains (Hof et al. 1998), have also been suggested to be important mechanisms to inhibit PTP activity. For receptor type PTPs, ligand-induced dimerization can result in inhibition of phosphatase activity of some enzymes like PTP- α , CD45 (Majeti et al. 1998), and possibly PTP- β/ζ (Deuel et al. 2002). Activation and alteration of subcellular localization patterns following calpain-mediated proteolytic cleavage is yet another means of regulation for intracellular PTP1B (Frangioni et al. 1993; Rock et al. 1997), PTPMEG (Gu and Majerus 1996; Pasquet et al. 1998), and more recently for the receptor type PTP- α and PTP- ϵ (Gil-Henn et al. 2001).

Type Prospirates Prospirates PTP Prospirates Prospirates PTP Prospirates Prospirates PTP Prospirates Prospirates PTP Prospirates Prospirates PTEN Prest Prest PTP diabetes, inacrophage activation, cytokine signaling Prest PTP elsetes, inacrophage activation, cytokine signaling Prest PTP elsetes, inacrophage activation, cytokine signaling Prest PSP Noonan and Leopard syndromes, myeloid leukemias Prest PSP Noonan and Leopard syndromes, myeloid leukemias Prest PAA syndrome (autoinfrarmatory disease) Prest Prest PAPA syndrome (autoinfrarmatory disease) Prest Prest PAPA syndrome (autoinfrarmatory disease) Prest Prest PAPA syndrome (autoinfrarmatory diseases) Prest Prest </th <th></th> <th>Ž</th> <th>Name</th> <th>Related Human diseases/therapeutic targets/physiological roles</th> <th>Proi</th> <th>Protein Structure</th> <th></th>		Ž	Name	Related Human diseases/therapeutic targets/physiological roles	Proi	Protein Structure	
Cdc25 various cancers, positively regulates cell cycle CH2PTPCH2 CH2 PTEN hamartonan-neoplasis syndrome PTP+C2 CH2 PTP1B diabetes, obesity, breast and ovarian cancers PTP+C2 CH2 PTP1B diabetes, obesity, breast and ovarian cancers PTP+C2 CH2 SHP-1 diabetes, macrophage activation, cytokine signaling PTP+C2 CH2 SHP-1 diabetes, macrophage activation, cytokine signaling PTP+C2 CH2 SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers PTP+C2 CH2 SHP-2 Noonan and Leopard syndromes, myeloid leukemias PTP+C2 CH2 SHP-2 Noonan and Leopard syndromes, myeloid leukemias PTP+C2 CH2 PTP PAA syndrome (autoinflammatory disease) PTP+C2 CH2 CH2 PTP PAA syndrome (autoinflammatory disease) PTP+D1 CH2 CH2 CH2 PTP PAA syndrome (autoinflammatory disease) PTP+D1 CH2	ммл		MW	positively regulates growth factor and adhesion signaling	- PTP-		
PTEN Imamatroma-neoplasia syndrome PTP-ICA PTP1B diabetes, obesity, breast and ovarian cancers PTP-ICA TCPTP diabetes, obesity, breast and ovarian cancers PTP-ICA TCPTP diabetes, inacrophage activation, cytokine signaling PTP-ICA SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers PTP-ICA SHP-2 Noonan and Leopard syndromes, myeloid leukemias PTP-ICA PTP PHP-SH-SH-SH-SH-SH-SH-SH-SH-SH-SH-SH-SH-SH-	49	0	Cdc25	various cancers, positively regulates cell cycle	CH2 PTP CH2-		_
PTP1B diabetes, obesity, breast and ovarian cancers PTP1B diabetes, obesity, breast and ovarian cancers TCPTP diabetes, macrophage activation, cytokine signaling PTP1B PTP1B SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers PTP1B PTP1B SHP-2 Noonan and Leopard syndromes, myeloid leukemias PTP1B PTP1B PTP- PAPA syndrome (autoinflammatory disease) PTP1B PTP1B PTP- PAPA syndrome (autoinflammatory disease) PTP1B PTP1B PTP- PAPA syndrome (autoinflammatory disease) PTP1B PTD1B PTP- PTP2 PaPA syndrome diseases, antibody-based therapy PTD1B PTD1B PTP- PTP2 PTP1B PTD1B PTD1B PTD1B PTP- PTP2 PTD20(cth) and beta-catenin regulation PTD1B PTD1B PTD1B PTP3 PTD20(cth) and beta-catenin regulation, vascular developm	Da	•	PTEN	hamartoma-neoplasia syndrome	— РТР C2>●		
TCPTP diabetes, macrophage activation, cytokine signaling Proprint SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers Proprint SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers Proprint SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers Proprint SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers Proprint SHP-1 leukemias, breast, ovarian, prostate and pancreatic cancers Proprint SHP-1 Proprint State PTP- PAPA syndrome (autoinflammatory disease) PAPA syndrome (autoinflammatory disease) Proprint Propri			PTP1B		010	Prolin-rich motif	
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Cy SHP-2 Nonan and Leopard syndromes, myeloid leukemias Purperperion PTP- PAPA syndrome (autoinflammatory disease) PTP- PTP- PTP PAPA syndrome (autoinflammatory disease) PTP- PTP- PTP Severe combined immunodeficiency disease (SCID), PTP- PTP- PTP- PTP CD45 autoimmune diseases, antibody-based therapy PTP- PTP- PTP- PTP PTP PTP PTP PTP- PTP- PTP- PTP- PTPs colon cancer, diabetes, positively regulates signaling via Src PTP- PTP-<		loso	SHP-1	leukemias, breast, ovarian, prostate and pancreatic cancers		transmembrane	
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CD45 severe combined immundeficiency disease (SCID), autoimmune diseases, antibody-based therapy PTP PTP <th>ITP (I</th> <th></th> <th>PTP- PEST</th> <th>PAPA syndrome (autoinflammatory disease)</th> <th>-втр-б-б-б-</th> <th>٦</th> <th>like</th>	ITP (I		PTP- PEST	PAPA syndrome (autoinflammatory disease)	-втр-б-б-б-	٦	like
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LAR diabetes, neuronal development and regeneration PTPo neuronal development and regeneration PTPa neuronal development and regeneration PTPa learning and memory, neurite outgrowth DEP1 p120(cth) and beta-catenin regulation, vascular development PTPp neuronal migration, neurite outgrowth, gliogenesis	ifice	<u>م</u> لاد	PTP_{E}	positively regulates signaling via Src phosphorylation		0	
PTP- PTP- PTP- PTP- PTP3 neuronal development and regeneration - PTP- PTP- PTP3 learning and memory, neurite outgrowth - PTP- PTP- DEP1 p120(ctn) and beta-catenin regulation, vascular development - PTP- PTP- PTP3 neuronal migration, neurite outgrowth, gliogenesis - PTP- PTP-	ds-J	l rot	LAR	diabetes, neuronal development and regeneration			
PTPδ learning and memory, neurite outgrowth DEP1 p120(ctn) and beta-catenin regulation, vascular development PTPβ neuronal migration, neurite outgrowth, gliogenesis	ρŢλι	dəc	ΡΤΡσ	neuronal development and regeneration	-ртр-ртр-		
p120(ctn) and beta-catenin regulation, vascular development neuronal migration, neurite outgrowth, gliogenesis		эЯ	ΡΤΡδ	learning and memory, neurite outgrowth			
neuronal migration, neurite outgrowth, gliogenesis			DEP1	p120(ctn) and beta-catenin regulation, vascular development			
			ртрβ	neuronal migration, neurite outgrowth, gliogenesis			



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2.4 Involvement of PTP in Human Diseases

The physiological functions of PTPs have primarily been derived from genetargeting studies in mice or genetic studies involving human diseases (Li and Dixon 2000). It is easily noted that PTPs are involved in a variety of biological functions that include embryonic development, hematopoiesis, immunity, metabolism, and others (Fig. 1). It stands to reason that although PTPs are affected in several genetic and metabolic diseases, not all can serve as therapeutic targets. For example, those diseases that are caused by a loss of function may mainly be targeted using gene therapy approaches (i.e., PTEN and SHP-1, see below). A caveat would be that in dominant-negative mutations, gene therapy would unlikely be usable. Other approaches that could specifically target the mutated PTPs, such as antisense or siRNA, could then serve as potential therapeutic treatment.

First, we discuss below PTP1B in diabetes and obesity as a paradigm, and the following will summarize recent findings that demonstrate a relevant role of various PTPs in specific human disease applications. For some, we will also reveal their current state as therapeutic targets either for PTP candidates for pharmaceutical inhibition or as potential targets for gene therapies.

2.4.1 Metabolic Diseases

2.4.1.1 PTP1B

Undeniably, PTP1B has been the most studied PTP to date as judged by the recent numerous publications and its potential impact in the diabetes field. The intense focus on PTP1B is primarily based on the observation that PTP1B is a negative regulator of insulin and leptin signal transduction (Cheng et al. 2002a; Cook and Unger 2002). Indeed, PTP1B-deficient mice display both increased insulin (Elchebly et al. 1999a; Klaman et al. 2000b) and leptin signaling (Cheng et al. 2002b; Zabolotny et al. 2002). Furthermore, PTP1B may also modulate the metabolic functions of growth hormone (Gu et al. 2003). Importantly, these mice are resistant to diabetes obesity.

The direct implication of PTP1B in these human diseases is further fortified by the identification of associated mutations and polymorphisms in the human PTP1B gene. The PTP1B locus maps to human chromosome 20 in the region q13.1-q13.2 (Brown-Shimer et al. 1990) and its mouse ortholog to the syntenic H2-H3 region of chromosome 2 (Forsell et al. 2000). Interestingly, this region was also identified as a quantitative trait loci (QTL) linked to insulin and obesity (Lembertas et al. 1997). Consistent with a role for PTP1B in human insulin resistance, single nucleotide polymorphisms have been found within the coding region (Echwald et al. 2002; Mok et al. 2002) or the 3' untranslated region (UTR) (Di Paola et al. 2002), both of which are associated with diabetic parameters.

The identification of PTP1B as a critical regulator of insulin action has led to an explosive motivational force to generate PTP1B inhibitors. It is not surprising that there exist a number of approaches for developing anti-PTP compounds. Such ongoing programs are presently examining the efficacy of small-molecule inhibitors directed against the catalytic site, antisense oligonucleotides, monoclonal antibodies and gene therapy. It is now accepted that inhibition of PTP1B represents the best paradigm for the development of PTP inhibitors in the treatment of type 2 diabetes and obesity. Considerable efforts to derive small chemical inhibitors of PTP1B guided by the information acquired on structural biology were initiated with some successes (reviewed in Johnson et al. 2002 and the chapter by Møller et al. in this volume).

One of the driving forces in the PTP field stems from the early observations that vanadate, a competitive inhibitor of PTPs, possesses insulin-mimetic properties. However, vanadate is non-specific in its action towards PTPs, and it presents toxic effects as it accumulates in the body. Novel PTP1B inhibitors have been designed using multiple approaches, and include both phosphorus and non-phosphorus derivatives. To date, the most potent PTP1B inhibitor published possesses a K_i of 2.4 nM (Zhang et al. 2002). This compound has also been shown to increase insulin receptor (IR) phosphorylation of hepatoma cells when used at the micromolar range. Currently, the major challenge in developing specific PTP1B small-molecule inhibitors is to find selectivity against T cell (TC)-PTP, the most closely related PTP, and the identification of suitable physico-chemical properties for oral delivery.

An alternative approach to small molecule inhibitors is the technology of antisense oligonucleotides (ASO). As a proof-of-concept demonstration, Abbott Laboratories and ISIS pharmaceuticals recently utilized PTP1B ASO in mouse models. The injection of a PTP1B ASO (ISIS-113715) in hyperglycemic obese mice was recently reported to improve insulin sensitivity (Zinker et al. 2002) and to modulate fat storage and lipogenesis in adipose tissue (Rondinone et al. 2002). Administration of their ASO was able to decrease PTP1B expression in both adipose and liver tissue. The resulting treated mice displayed increased insulin sensitivity and reduction in adiposity. Phase I clinical trials were announced last spring (2003).

Although PTP1B clearly plays a negative role in insulin signaling, its effects seem to be tissue specific. In particular, IR phosphorylation in adipose tissue seems unperturbed in PTP1B-deficient mice. This raises the possibility that other PTPs such as PTP- α and leukocyte antigen-related PTP (LAR),

may participate in this process (Cheng et al. 2002a). For example, LAR knockout mice exhibit alterations in glucose metabolism, albeit with only modest increases in IR phosphorylation (Ren et al. 1998). In contrast, muscle-specific transgenic expression of LAR in mice does seem to suggest that this enzyme may play a causative role in insulin resistance that is observed in diabetes (Zabolotny et al. 2001). In recent reports, TC-PTP has also been found to dephosphorylate the IR (Galic et al. 2003). Confirmation of this finding in animal studies is ongoing.

2.4.2 Cancer

Intuitively, since the majority of oncogenes are tyrosine kinases, it was previously thought that PTPs might act as tumor suppressors. However, it is now clear that PTPs may act negatively or positively in modulating signaling pathways. In the past 5 years several examples of both type of action by PTPs in cancer have been reported, leading to the possible validation of some of those PTPs as genuine targets in cancer treatment. The following are some of the most obvious examples.

2.4.2.1 CDC25

The dual-specific phosphatase CDC25 members, CDC25A, -B and -C, are all known to act as positive regulators at distinct phases of the mammalian cell cycle through their ability to dephosphorylate and activate cyclin-dependent kinases (CDKs) (Nilsson and Hoffmann 2000). CDC25A is likely to be important for G1/S phase transition, in preserving genomic integrity and initiation of mitosis, while CDC25B and CDC25C act on the G2/M transition phase (Lazo et al. 2002). CDC25A and CDC25B possess oncogenic properties (Galaktionov et al. 1995), are transcriptional targets of the c-myc proto-oncogene (Galaktionov et al. 1996), and are overexpressed in various human tumors (Cangi et al. 2000; Pestell et al. 2000). Already, they have been reported to be important genes in prognostic studies, where expression of both CDC25A and CDC25B correlate with survival in patients suffering from ovarian cancers (Broggini et al. 2000). On the other hand, despite the biochemical evidence that implicates CDC25C in proliferation and transformation, CDC25C-deficient mice do not display an overt phenotype (Chen et al. 2001). Perhaps functional redundancy exists among the three family members.

One attractive aspect of CDC25 inhibitor development lies in the unique structural features of these enzymes compared to other PTPs. The crystal structures of CDC25A and CDC25B have both been solved, and show that most of the similarity to other PTPs is found in the active site loop contain-

ing the PTP signature motif. Focusing on diverging motifs may facilitate the design of specific inhibitors of the CDC25 PTPs. Furthermore, very little similarity exists between the three family members, and this may allow for an additional level of specificity.

Most CDC25 inhibitory compounds reported to date have been derived from both synthetic and natural products (Pestell et al. 2000). In particular, a vitamin K analog (a quinine derivative) has recently been shown to be a potent CDC25 inhibitor, and possesses anti-proliferative properties when administered to breast cancer and hepatoma cell lines (Kar and Carr 2000; Kar et al. 2003). This compound acts in the micromolar range, and thus more studies will likely be needed to increase effectiveness.

2.4.2.2 PTEN

PTEN (also called MMAC1 or TEP1) is a member of the dual-specific PTPs. PTEN was originally identified as a candidate tumor suppressor gene located on human chromosome 10q23. Recent studies have demonstrated that somatic mutations in PTEN are responsible for hamartoma-neoplasia syndrome such as Cowden, Bannayan-Zonana, and Lhermitte-Duclos diseases (Wishart and Dixon 2002). Although PTEN possesses both protein and lipid phosphatase activities, it preferentially dephosphorylates phospholipids. It is also its lipid phosphatase activity that is essential for PTEN tumor suppression (Maehama et al. 2001). Among D3-phosphorylated inositol phospholipids, PtdIns(3,4,5)P3 has been shown as the principal physiological substrate of PTEN, which regulates Akt and PDK1 activity.

2.4.2.3

Other PTPs Involved in Cancer Development

One promising target reported in the PTP family is the dual phosphatase PRL-3 and its closely related homolog PRL-1. These PTPs have been shown to promote invasiveness of cancer cells in vitro, and PRL3 was also found in metastatic human liver tumors (Saha et al. 2001; Zeng et al. 2003). Thus, targeting of PRL3 may provide an excellent means to prevent the development of metastasis.

SHP-1 is a SH2 domain-containing cytoplasmic PTP, and it is predominantly expressed in hematopoietic cells. SHP-1 has been suggested as a tumor suppressor and its expression is dramatically decreased in most lymphocytic-related cancers, as well as in breast, ovarian, prostate, and pancreatic cancers (Wu et al. 2003). SHP-2 is a close relative of SHP-1 and may also play an important role in cancer. Mutations in this gene are found in myeloid leukemias such as non-syndromic juvenile myelomonocytic leukemia (Tartaglia et al. 2003). Unlike the PTPs mentioned above, the evidence for the role of other PTPs in malignant transformation is less substantial. However, loss of heterozygosity (LOH) for a few PTPs has been demonstrated in various types of cancers. As mentioned above, LOH for SHP-1 occurs with high frequency in lymphoblastic leukemias (Oka et al. 2002). PTP- κ , whose expression is developmentally regulated in the CNS, was recently shown to exhibit LOH in 76% of CNS lymphomas (Nakamura et al. 2003). DEP1 is a candidate for the mouse colon-cancer susceptibility locus *Scc1* and is frequently deleted in human cancers (Ruivenkamp et al. 2002).

PTP-PEST is a cytosolic enzyme that was found to modulate the actin cytoskeleton and focal adhesion turnover (Angers-Loustau et al. 1999; Garton and Tonks 1999). Following fibronectin signaling activation, a cascade of tyrosine phosphorylation events is initiated that cumulate into the tyrosine phosphorylation of the adaptor protein p130cas. From this protein, several signals branch out that promote actin polymerization to cell proliferation. By inhibiting PTP-PEST activity, one may thus block cell migration as was shown with the knockout PTP-PEST fibroblasts (Angers-Loustau et al. 1999). Hence, specific inhibitors of PTP-PEST could serve as anti-metastatic agents and anti-angiogenic agents.

On the other hand, PTPs can also play a positive role in cellular transformation. PTP- α is a receptor PTP that activates Src kinases via dephosphorylation of the C-terminal negative regulatory site. Overexpression of PTP- α causes cellular transformation of fibroblasts via activation of Src kinases (Zheng et al. 1992; den Hertog et al. 1993). Conversely, in fibroblasts lacking PTP- α , both Src and Fyn activation are diminished under resting conditions as well as during integrin signaling (Ponniah et al. 1999; Su et al. 1999). In human colon cancer, increased expression of PTP- α has been observed (Tabiti et al. 1995). PTP- ϵ , which is closely related to PTP- α , also has been suggested to activate Src kinase. It has been shown that absence of PTP- ϵ reduced Src activity and altered Src phosphorylation in neu-induced mammary tumor cells (Gil-Henn and Elson 2003). Interestingly, increased Src expression and activity are also increased in colon cancer, suggesting a possible link. On the same theme, PTP1B has been reported to similarly dephosphorylate the negative regulatory site of c-src (Arregui et al. 1998; Bjorge et al. 2000; Cheng et al. 2001). Moreover, PTP1B was reported to be increased in expression in breast and ovarian cancers (Wiener et al. 1994a,b; Zhai et al. 1993). These findings suggest that in many instances selectable inhibition of PTP activity would be a suitable means for cancer treatment.

2.4.3 Noonan Syndrome and SHP-2

SHP-2 is a widely expressed non-receptor PTP that contains two amino-terminal SH2 domains, a carboxyl-terminal catalytic domain and a C-terminal segment containing two tyrosine phosphorylation sites (Feng 1999). Several studies to delineate the function of SHP-2 in cell signaling demonstrate that it primarily acts as a positive regulator of the cellular response to growth factors, hormones, cytokines, and cell adhesion molecules, and thus it is a critical regulator of proliferation and differentiation. However, it can act as a negative regulator in some pathways. Recently, it has been shown that germline mutations in PTPN11, which encodes SHP-2, cause Noonan syndrome and the related Leopard syndrome, both sharing several clinical features such as facial dysmorphia, short stature, and cardiac defects (Tartaglia et al. 2001; Digilio et al. 2002; Legius et al. 2002; Tartaglia et al. 2002). As mentioned above, mutations in SHP-2 also can lead to myeloid leukemias (Tartaglia et al. 2003). Interestingly, these mutations are suggested to result in a gain of function of SHP-2 where the intramolecular inhibitory binding of the amino-SH2 domain would be lost, resulting in a constitutive activation of the phosphatase. SHP-2 may be also important in pathogenesis by Helicobacter pylori (see Sect. 2.4.6). These findings support the potential value for SHP-2 specific inhibitors as therapeutic agents. In contrast with gain of function, SHP-2 gene knockout mice show embryonic lethality at midgestation and data from SHP-2 gene knockout and SHP-2 chimeric mice clearly indicate that this PTP is essential for hematopoiesis and multiple embryonic development.

2.4.4 Neuronal Development and Nerve Regeneration

The closely related receptor type PTPs, LAR, PTP- δ and PTP- σ have been shown to play crucial roles in axon growth and guidance (Johnson and Van Vactor 2003). The extracellular segment of these proteins consists of three immunoglobulin-like repeats and four to eight type III fibronectin repeats generated by alterative splicing. These structures resemble cell adhesion molecules (CAMs) and they are specifically expressed in the CNS, suggesting cellular roles during axon guidance and synapse formation. Consistent with this notion, *Drosophila* mutants in the closely related receptor-type PTP (RPTP) (Dlar and DPTP69D) exhibit severe defects in axonal pathfinding (Johnson and Van Vactor 2003).

Genetic studies in mice have strongly implicated these enzymes in the development of the nervous system (Yeo et al. 1997; Elchebly et al. 1999; Wallace et al. 1999; Uetani et al. 2000). Recently PTP- σ has been shown as a negative modulator of neurite outgrowth, and PTP- σ knockout mice functionally recover faster than wild-type mice after facial and sciatic nerve axotomy in vivo (McLean et al. 2002; Thompson et al. 2003). By comparison, the absence of LAR delays recovery of sensory, but not of motor, nerve function following sciatic nerve injury (Xie et al. 2001). Furthermore, the extracellular domains of LAR and PTP- δ function as a homophilic ligand and

promote neurite outgrowth (Wang and Bixby 1999; Yang et al. 2003). These data suggest that targeting these PTPs may achieve an improvement in nerve regeneration after neuronal injuries.

Another enzyme involved in human neuronal diseases is the DSP Laforin. Positional cloning identified this gene in Lafora's disease, an autosomal progressive myoclonus epilepsy (Minassian et al. 1998; Serratosa et al. 1999). Genetically engineered mice deficient for Laforin show most of the symptoms of Lafora's disease, and may provide a model to understand the cellular mechanisms of Lafora's disease (Ganesh et al. 2002).

2.4.5 PTPs and Immunomodulation

2.4.5.1 CD45

CD45 is the prototypical transmembrane PTP, and is highly expressed in all hematopoietic lineages (Sasaki et al. 2001). It is well established that CD45 is critical for T and B cell activation, primarily via activation of the Src kinase Lck (Thomas 1999). Under resting conditions, Lck is maintained in an inactive state via intramolecular interactions between its C-terminal phosphorylated tyrosine (Y505) and its SH2 domain. During TCR engagement, CD45 dephosphorylates Y505 on Lck, thus allowing for activation of kinase activity. Activated Lck then phosphorylates its substrates to transmit the TCR signals that ultimately lead to transcription of genes that are important for T cell function, such as interleukin-2.

Mice lacking CD45 demonstrate impaired T cell differentiation and activation as well as B cell activation and are therefore severely immunodeficient (Kishihara et al. 1993; Byth et al. 1996). Two recent descriptions of clinically relevant CD45 mutations associated with severe combined immunodeficiency disease (SCID) in humans correlate well with the phenotype reported for the CD45-deficient mice (Kung et al. 2000; Tchilian et al. 2001). In those cases, mutations of CD45 confirm that this PTP serves as a modifier gene in immunodeficiency.

As a therapeutic target, anti-CD45 antibodies have demonstrated an antileukemic effect when used either unconjugated or attached to radioactive iodine (Nemecek and Matthews 2002). For example, ¹³¹I-labeled anti-CD45 antibodies have been used to deliver radiation directly to leukemic cells, because CD45 is found on cell surface isolated from most of leukemia patient samples (Countouriotis et al. 2002). Clinical trials of this antibody-based therapy using anti-CD45 antibodies are currently in phase I/II (www.clinicaltrials.gov).

CD45 is also presumed to be a good immunomodulatory molecule to promote transplantation tolerance based on its accessibility as a transmembrane protein (Fecteau et al. 2001; Ko et al. 2002; Pagel et al. 2002). Allograft rejection is a T cell-dependent process, and interference with T cell activation through T cell receptor (TCR) can induce anergy in CD4⁺ T cell clones. Indeed, in this context, allograft rejection has been shown to be suppressed using an antibody against CD45RB isoforms (Lazarovits et al. 1996). One possible mechanism by which such an interaction induces allograft tolerance involves the upregulation of CTLA4 (cytolytic T lymphocyte-associated antigen 4) which is an important T cell down-regulatory molecule (Fecteau et al. 2001).

Very recently, it has been shown that 77 C/G mutation in the human CD45 gene, which results in increasing CD45RA isoform expression on cell surface, is linked to several autoimmune diseases such as systemic sclerosis and hepatitis (Vogel et al. 2003), suggesting CD45 inhibitors and/or antibodies are potent therapeutics for those autoimmune diseases.

2.4.5.2 Other PTPs in the Immune System

Since the discovery that loss-of-function mutations in SHP-1 are responsible for systemic autoimmunity and severe inflammation observed in both *motheaten* and *viable motheaten* mice, SHP-1 has been shown as a major negative regulator of various signaling pathways, including T and B cell antigen receptors in hematopoietic cells (Zhang et al. 2000). Although SHP-1 has been strongly suggested to associate with various cancers and infectious diseases (see also Sects. 2.4.2 and 2.4.6), further investigation of its pathogenic roles in those diseases is proposed to develop therapeutic agents for SHP-1 associated diseases.

PTP-PEST is also highly expressed in hematopoietic cells (Cote et al. 2002). Recent work by Davidson and Veillette (2001) indicates that it is an important negative modulator of lymphocyte activation. In addition, an exciting report by Wise et al. (2002) pointed to the lack of PTP-PEST association with the adaptor protein proline serine threonine phosphatase-interacting protein (PST-PIP) as the cause of pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome, an autoinflammatory disorder.

2.4.6 PTPs in Infectious Diseases

Microbial pathogens are responsible for a large spectrum of diseases that are leading causes of morbidity and mortality worldwide. Of interest are the pathogens expressing virulence factors that block animal defense reactions or alter host cell function through modulating tyrosine phosphorylation to facilitate their life cycle. Several also express their own PTPs that are essential for their infectious properties. It stands to reason that focusing on inhibiting those PTP may be a new approach in blocking these infectious agents.

2.4.6.1 Salmonella

Salmonellosis has been known as a foodborne illness, and emergence of multi-drug resistant strain is threatening to become a serious public health problem. *Salmonella* expresses a DSP (named Sptp) that participates in the entry process of this pathogen into mammalian cells that are normally non-phagocytic. By inducing a phagocytic behavior, this allows the bacterium to be protected from the host immune system (Finlay and Cossart 1997). Sptp is a multidomain protein that includes a GTPase activating domain and a dual specificity PTP domain. Although little is known about the PTP domain, it seems to play a role in downregulating the MAPK pathway (Murli et al. 2001). This may act as a means to shut-off host cell functions.

2.4.6.2 Yersinia pestis

Yersinia pestis presents historical value as being responsible for the Black Plague (Fallman et al. 2002). This pathogen expresses the phosphatase YopH that contributes to block macrophage phagocytosis and slow down the onset of the inflammatory response, thus favoring pathogen survival. The activity of YopH is essential for this effect, and it suggests that dephosphorylation of host proteins participates in this mechanism. These substrates include p130Cas and Fyn binding protein. Recently, aurintricarboxylic acid has been shown as a selective YopH inhibitor, and it may be therapeutically useful (Liang et al. 2003).

2.4.6.3 Helicobacter pylori

Helicobacter pylori is a gram-negative bacterium thought to be a primary cause of gastric ulcers and cancer. Within its arsenal, the CagA and VacA proteins bind host PTPs, and these events are critical for its detrimental effects. CagA is injected into host cells and perturbs cellular functions, including the induction of morphological changes. One target of CagA has been identified as SHP-2, and disruption of this complex abolishes the effects of CagA (Higashi et al. 2002). Hence, the formation of this complex also stimulates the phosphatase activity of SHP-2. On the other hand, VacA is critical for causing massive vacuolization of the host cell, and recently this process has been shown to be dependent on its ability to bind the receptor phosphatase activity for FIP- ζ in a ligand-like fashion (Fujikawa et al. 2003).

2.4.6.4 Other PTPs in Infectious Diseases

Leishmania donovani (Ld) is an intracellular protozoan parasite that is the causative agent of visceral leishmaniasis. Recent findings suggest that the capacity of Ld to suppress host immune responses such as macrophage phagocytosis is related to SHP-1 (Blanchette et al. 1999; Nandan et al. 2002). It has also been suggested that calpain-mediated cleavage and activation of host cell PTP1B may play a role in amebiasis caused by the protozoan *Entamoeba histolytica* (Teixeira and Mann 2002). It is well known that the transcription and replication of HIV-1 (human immunodeficiency virus 1), the causal agent of acquired immunodeficiency syndrome (AIDS), is linked to T cell activation. Interestingly, SHP-1 may act as an important host factor participating in HIV-1 long terminal repeat (LTR) transactivation (Fortin et al. 2001; Ouellet et al. 2003).

3 Conclusion

From the initial PTP studies in the early 1980s (Nelson and Branton 1984; Sparks and Brautigan 1986), to the successful purification of PTP1B (Tonks et al. 1988a,b), we have gathered an impressive knowledge on the enzymatic properties and structure of PTPs. The realization that PTP1B could potentially be an outstanding target for diabetes and obesity has launched a broad search for pharmacological inhibitors. For the past 5 years this search revealed several difficulties in finding the optimal compounds. In spite of the difficult chemistry, one may hope that these efforts will continue. As this review entails to show, a great number of other targets await the first success with PTP1B. Once an optimal compound for PTP1B is found, it would be much easier to model such a chemical(s) to the other very closely related members of the family (Andersen et al. 2001). Overall, it is clear that the PTP family holds great promise for the treatment of several human diseases, and we only need the chemistry to catch up with the outstanding biology that has been recently revealed on many of the PTP family members.

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Structure-Based Design of Protein Tyrosine Phosphatase Inhibitors

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Abstract Protein tyrosine phosphatases (PTPs) are a family of intracellular enzymes that remove phosphate from tyrosine phosphorylated proteins. The PTP superfamily includes tyrosine phosphate-specific classical PTPs, dual-specificity PTPs, and low-molecular-weight PTPs. PTPs and protein tyrosine kinases reversibly regulate the phosphotyrosine level in selected cellular proteins, thereby controlling many important signaling pathways in eukaryotes. Aberrant tyrosine phosphorylation levels have been associated with the development of cancer, autoimmunity, and diabetes, thus indicating that PTPs might play

important etiological and pathogenic roles in these diseases. As a result, these enzymes have recently attracted much interest as potential drug targets. This is in particular due to the finding that PTP1B knockout mice show increased insulin sensitivity and resistance to diet-induced obesity, thus indicating that PTP1B is an important negative regulator of insulin and leptin action and hence a potentially important drug target for the treatment of diabetes and obesity. The development of PTP inhibitors, in particular PTP1B inhibitors, has been greatly facilitated by an impressive number of X-ray structures that have allowed structure-based design of highly selective inhibitors of PTP1B, the main focus of this review. The initial attempts to design selective PTP inhibitors were based on replacement of pTyr with non-hydrolyzable phosphotyrosyl mimetics in small, efficient PTP peptide substrates, thereby utilizing both the potency and selectivity provided by the amino acid residues. However, several groups have now shown that it is possible to synthesize highly potent and selective non-phosphorus, non-peptide inhibitors of PTP1B. At this point, these achievements to some extent seem to have been reached at the expense of appropriate pharmacokinetic properties, including cellular uptake. Therefore, the next wave within the field of PTP inhibitors is likely to be focused on improvements in this respect. In addition, several other PTPs could potentially be attractive drug targets in autoimmunity and cancer.

Keywords Protein tyrosine phosphatase · PTP1B · X-ray · Bioinformatics · Diabetes mellitus · Enzyme kinetics

1 Introduction

Protein tyrosine phosphatases (PTPs) are a family of intracellular enzymes that remove phosphate from tyrosine phosphorylated proteins. The PTP superfamily includes the following three families: (1) tyrosine phosphate-specific, classical PTPs, (2) dual-specificity PTPs and (3) low-molecular-weight PTPs. Together with tyrosine kinases that catalyze the addition of phosphate to tyrosine residues, PTPs reversibly regulate the phosphotyrosine (pTyr) level in selected cellular proteins, thereby controlling many important signaling pathways in eukaryotes. (For recent reviews, see Møller et al. 2000; Johnson et al. 2002; van Huijsduijnen et al. 2002; Tonks 2003; Zhang and Lee 2003.) In addition to protein substrates, a small number of the PTP superfamily members, including PTEN and myotubularin, dephosphorylates lipid substrates (Maehama et al. 2001; Wishart and Dixon 2002). Traditionally, the classical PTPs (Fig. 1), which are the main topic of the current review, have been classified as receptor-type (RT1-8) or non-transmembrane/intracellular (NT1-9) PTPs based on their non-catalytic domain structure and the presence or absence of a transmembrane region (Fischer et al. 1991; Tonks and Neel 2001). We have recently performed a phylogenetic classification of PTPs and shown that there is a very close relationship between PTP domain sequence similarity and functional domains in the full-length proteins (Andersen et al. 2001). Thus, receptor-type PTPs, previously classified into

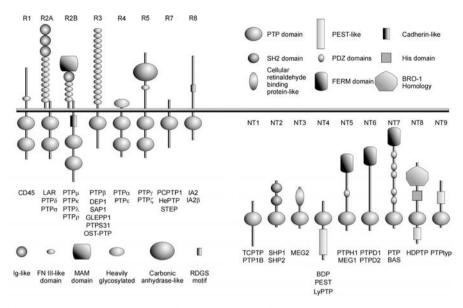


Fig. 1 Schematic representation of the classical PTP family

nine distinct subtypes, were categorized into virtually identical groups to these based on sequence homology of the catalytic domains. Similar observations and categorizations were made for intracellular PTPs.

The catalytic domains of classical PTPs consist of ~230 amino acid residues. Based on amino acid comparisons, 10 discrete and highly conserved motifs can be identified in these domains (Andersen et al. 2001). The active site sequence His-Cys-(Xxx)₅-Arg-Ser/Thr (residues 214–222; PTP1B numbering, which is used throughout) defines the PTP family and is often referred to as the PTP loop, the P-loop or the PTP signature motif.

Due to the critical role of PTPs in controlling cellular processes such as growth and differentiation, cell cycle, and metabolism, these enzymes have recently attracted much interest as potential drug targets. As a result, a number of laboratories both in academia and the pharmaceutical industry have devoted much energy to the discovery and development of selective inhibitors of this enzyme class. In the following we will review the most recent progress in the field and present the strategy and technological platform developed in house by the PTP inhibitor group at Novo Nordisk. From the outset it should be emphasized that we will restrict our discussion to compounds that are reversible, competitive, and time-independent PTP inhibitors and for which detailed enzyme kinetic analyses and/or structural information are provided, i.e., compounds that are suitable for structurebased design. There is a significant risk of identifying oxidizing or alkylating compounds when searching for PTP inhibitors (for details, see Sect. 8.1) and structure-based optimization on such compounds is often difficult. It should, however, be noted that compounds that irreversibly and/or more broadly in a time-dependent manner modify PTPs may still be useful in the clinic (Skorey et al. 1997; Pathak and Yi 2001; Pathak et al. 2002; Yi et al. 2002; Kar et al. 2003). We therefore refer the reader to the original literature or reviews that include information on such compounds, e.g., Ripka (2000), Urbanek et al. (2001), Blaskovich and Kim (2002), and Patankar and Jurs (2003).

2 The Catalytic Machinery

A detailed understanding of the three-dimensional geometry of the active site and the catalytic machinery is a prerequisite for structure-based design of active site-directed enzyme inhibitors. The PTP field had been advanced early on by a number of detailed enzyme kinetic and structural studies (reviewed in Burke and Zhang 1998; Denu and Dixon 1998). The X-ray structure of catalytically inactive PTP1B_{C2155} mutant complexed with a synthetic tyrosine phosphorylated hexapeptide revealed the architecture of the PTP signature motif Cys-(Xxx)₅-Arg that forms an efficient phosphate binding site. The peptide main chain of this motif forms a half-circle with Cys215 almost in the middle, and the phosphate oxygen atoms of pTyr form a number of hydrogen bonds to the main chain nitrogens and the guanidinium group of Arg221 (Jia et al. 1995) (see Table 1 for a comprehensive list of X-ray structures of PTP1B). The binding of pTyr is further stabilized by hydrophobic and aromatic π - π interactions between the phenyl ring of pTyr and residues such as Tyr46 and Val49 in the active site pocket.

The hydrolysis of pTyr substrates is a two-step process. The consensus active site Cys215 plays an essential role in the first step. This cysteine has a low pK_a value (i.e., it is deprotonated and charged at physiological pH) and acts as a nucleophile (Pot and Dixon 1992b), which attacks phosphate leading to the formation of a cysteine-phosphate intermediate (Guan and Dixon 1991) (Fig. 2, parts 1-3). Binding of pTyr substrates to PTPs is followed by closure of the so-called WPD loop (residues 179-183) in a conformation that traps the pTyr substrate in the active site pocket. The closed WPD loop is stabilized by hydrogen bonds between the side chain of Arg221 and phosphate and the carbonyl group of Pro180 as well as by π - π interactions between Phe182 and the phenyl ring of pTyr. The closure of the WPD loop brings Asp181 into a favorable position relative to the scissile oxygen of pTyr to act as a general acid donating a proton to the leaving group (i.e., the substrate) (Zhang et al. 1994b; Denu et al. 1996; Lohse et al. 1997) (Fig. 2, parts 2 and 3). In the second step of catalysis, the thiol phosphate intermediate is attacked by a nucleophilic water molecule that is positioned optimally

Ligand No.	PDB ID	Ligand name	Reference	Ligand No.	PDB ID	Ligand name	Reference
	1AAX	1AAX Bis(<i>para</i> -phosphophenyl)methane	Puius et al. 1997	37	1L8G	7-(1,1-Dioxo-1H-benzo[d]isothiazol-3-yloxymethyl)- 2-(oxalyl-amino)-4,7-dihydro-5H-thieno[2,3-c]pyran- 3-carboxylic acid	Iversen et al. 2001
	1BZC	4-Carbamoyl-4-{[6-(difluoro- phosphono-methyl)-naphthalene- 2-carbonyl]-amino}-butyric acid	Groves et al. 1998	39	1LQF	N-Benzoyi'-1-glutamyl-[4-phosphono- (difluoromethyl)]-1-phenylalanine-[4-phosphono (difluoro-methyl)]-1- phenyl-alanineamide	Asante- Appiah et al. 2002
	1BZH	Fluoromalonyl tyrosine, (2-amino-2-carbamoyl-ethylsulfanyl)- acetic acid	Groves et al. 1998	40	1N6W	N-{1-[5-(1-Carbamoy]-2-mercapto-ethylcarbamoy])- pentylcarbamoy]]-2-[4-(difluoro-phosphono-methy])- pheny]]-ethyl]-3-{2-[4-(difluoro-phosphono-methy])- pheny]]-acetylamino}-succinamic acid	Sun et al. 2003
	1BZJ	6-(Difluoro-phosphono-methyl)- naphthalene-2-carboxylic acid	Groves et al. 1998	43	1NL9	2-{[4.(2-Acetylamino-2-pentylcarbamoyl-ethyl)- naphthalen-1-yl]-oxalyl-amino}-benzoic acid	Szczepan- kiewicz et al. 2003
33	1C83	6-(Oxalyl-amino)-1 <i>H</i> -indole-5- carboxylic acid	Andersen et al. 2000	44	INNY	3-({5-[(N-Acetyl-3-[4-[(carboxycarbonyl) (2-carboxyphenyl)amino]-1-naphthyl}-l-alanyl) aminolpentylloxy)-2-naphthoic acid	Szczepan- kiewicz et al. 2003
32	1C84	3-(Oxalyl-amino)-naphthalene-2- carboxylic acid	Andersen et al. 2000	42	1N06	2-[(Carboxycarbony])(1-naphthyl)amino]-benzoic acid	Szczepan- kiewicz et al. 2003
1	1C85	2-(Oxalyl-amino)-benzoic acid	Andersen et al. 2000		INWE	N-[4-(2-{2-[3-(2-Bromo-acetylamino)- propionylamino]-3-hydroxy-propionylamino}- ethyl)-phenyl]-oxalamic acid	Erlanson et al. 2003
36	1C86	2-(Oxalyl-amino)-4,7-dihydro-5H- thieno[2,3-c]pyran-3-carboxylic acid	Iversen et al. 2000	2	INWL	3-(4-[2-[2-(2-Bromo-acetylamino)-ethyldisulfanyl]- ethylcarbamoyl}-cyclohexyl-carbamoyl)pyrazine-2- carboxylic acid	Erlanson et al. 2003
36	1C87	2-(Oxalyl-amino)-4,7-dihydro-5H- thieno[2,3-c]pyran-3-carboxylic acid	Iversen et al. 2000	45	1NZ7	2-[(4-[2-Acetylamino-2-[4-(1-carboxy-3-methylsulfanyl- propylcarbamoyl)-butyl-carbamoyl]-ethyl}-2-ethyl- phenyl)-oxalyl-amino]-benzoic acid	Xin et al. 2003

Table 1 PTP1B X-ray structures

(
Ligand No.	PDB ID	Ligand name	Reference	Ligand No.	PDB ID	Ligand name	Reference
35	1C88	2-(Oxalyl-amino)-4,5,6,7-tetrahydro- thieno[2,3-c]pyridine-3-carboxylic acid	Iversen et al. 2000		10EM	Apo structure (sulfenyl-amide bond between C215Sg and S216 N)	Salmeen et al. 2003
	1ECV	5-Iodo-2-(oxalyl-amino)-benzoic acid	Andersen et al. 2000		10E0	Apo structure (C215 oxidized to cysteinesulfinic acid)	Salmeen et al. 2003
	IEEN	Phosphotyrosine, para-(benzoyl)- phenylalanine peptide, D-A-D-BPA-PTyr-I-I-P-Q-Q-G (C215S mutant)	Sarmiento et al. 2000		10ES	Apo structure (sulfenyl-amide bond between C215Sg and S216 N)	Salmeen et al. 2003
	1EEO	Phosphotyrosine peptide, L-E-L-E-F-PTyr-M-D-Y-E (C215S mutant)	Sarmiento et al. 2000		10ET	Apo structure (C215 oxidized to s-hydroxy-cysteine)	Salmeen et al. 2003
	1G1F	Phosphotyrosine peptide, Q-T-D-PTyr-PTyr-R (C215A mutant)	Salmeen et al. 2000		10EU	Apo structure (C215 oxidized to cysteine-s-dioxide)	Salmeen et al. 2003
	1G1G	Phosphotyrosine peptide, T-D-Y-PTyr-R (C215A mutant)	Salmeen et al. 2000		10EV	Apo structure (C215 oxidized to cysteinesulfinic acid)	Salmeen et al. 2003
	1G1H	Phosphotyrosine peptide, T-D-PTyr-PTyr-R-K-G (C215A mutant)	Salmeen et al. 2000		IONY	2-{[2-(2-Carbamoyl-viny])-4-(2-methane- sulfonylamino-2-pentylcarbamoyl-ethyl)-phenyl]- oxalyl-amino}-benzoic acid	Liu et al. 2003a
15	1G7F	2-{4-[(2S)-2-[({[(1S)-1-Carboxy-2- phenyl- ethyl]amino}carbonyl)amino]-3- oxo-3-(pentylamino)propyl]phen- oxy}malonic acid	Bleasdale et al. 2001		10NZ	2-[(7-Hydroxy-naphthalen-1-yl)-oxalyl-amino]- benzoic acid	Liu et al. 2003a
16	1676	 2-(Carboxymethoxy)-5-[(2S)-2-({{2S}}- Bleasdale 2- et al. 2001 [(3-carboxypropanoyl)amino]-3-phe- nyl-propanoyl]amino)-3-oxo-3-(penty- lamino)- propyl]benzoic acid 	Bleasdale et al. 2001		1PH0	2-{4-[2-(S)-Allyloxycarbonylamino-3-{4- [(2-carboxy-phenyl)-oxalyl-aminol-phenyl}- propionylaminol-butoxy} 6-hydroxy-benzoic acid methyl ester	Liu et al. 2003c

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Table 1	Table 1 (continued)	ued)					
Ligand No.	PDB ID	Ligand PDB Ligand name No. ID	Reference	Ligand No.	PDB ID	Reference Ligand PDB Ligand name No. ID	Reference
	1GFY	1GFY 2-(Oxalyl-amino)-4,7-dihydro-5H- thieno[2,3-c]thiopyran-3-carboxylic acid	Peters et al. 2000		1PTY	1PTY Phosphotyrosine (C215S mutant)	Puius et al. 1997
	1157	Apo structure (C215S mutant)	Scapin et al. 2001	40	HX41	IPXH N-{1-[5-(1-Carbamoy]-2-mercapto-ethylcarbamoyl)- pentylcarbamoyl]-2-[4-(diffuoro-phosphono-methyl)- phenyl]-ethyl]-3-[2-[4-(diffuoro-phosphono-methyl)- phenyl]-acetylamino}-succinamic acid	Sun et al. 2003
					IQIM	5-{2-Fluoro-5-{3-{3-bydroxy-2-methoxy-carbony}- phenoxy)-propenyl]-phenyl}-isoxazole-3-carboxylic acid	Liu et al. 2003b

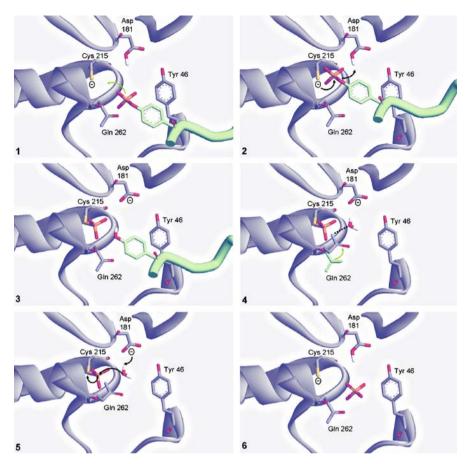


Fig. 2 Schematic representation of the catalytic mechanism of PTPs.1–3: Cys215 in the P-loop (PTP1B numbering used throughout) acts as a nucleophile, which attacks the phosphate leading to the formation of a cysteine-phosphate intermediate. In this first step of catalysis, Asp181 in the WPD loop acts as a general acid donating a proton to the leaving group. 4–6: In the second step of catalysis, the thiol-phosphate intermediate is attacked by a water molecule that is positioned optimally by Gln262

by Gln262 (Pannifer et al. 1998), hence leading to the release of phosphate (Fig. 2, parts 4–6). The understanding of the catalytic machinery in PTPs has been greatly facilitated by several seminal mutational and X-ray studies (Barford et al. 1994; Stuckey et al. 1994; Jia et al. 1995; Schubert et al. 1995; Fauman et al. 1996; Pannifer et al. 1998; Sarmiento et al. 1998).

3 Bioinformatics

Bioinformatics plays an important role in modern structure-based drug design. To assist our design of selective inhibitors, we have explored the human genome for its content of PTP genes and analyzed their primary amino acid sequence in context of X-ray crystal structure information. Specifically, the degree of PTP sequence conservation was calculated from a multiple sequence alignment of 37 different PTP domains and visualized in 3D to identify unique combinations of residues in the proximity of the active site that could be addressed in structure-based design of selective inhibitors (Iversen et al. 2000; Andersen et al. 2001). Four areas seemed particularly useful, since they could be targeted by low molecular active site-directed compounds (residues by PTP1B numbering): (1) 47-48; (2) 258-259; (3) 24-254 [i.e., the second aryl phosphate binding site (Puius et al. 1997)]; and (4) 116-118 (Fig. 3). In addition, the WPD loop may offer further possibilities for obtaining both potency and selectivity. Although no single residue in these areas appears to be a unique hallmark of any PTP, the combination of the residues in these areas represents important selectivity determining regions. We have in particular concentrated our efforts around utilizing the area defined by residues 47-48 and 258-259, as will be illustrated below (Sect. 9.2).

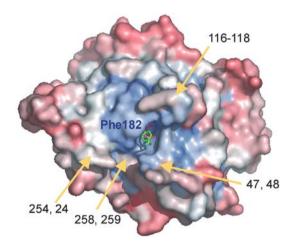


Fig. 3 Four selectivity determining regions visualized in 3D—useful for structure-based design of active site-directed inhibitors: (1) residues 47–48; (2) residues 258–259; (3) residues 24 and 254; and (4) residues 116–118. Phe182 (in the WPD loop) and a substrate (phosphotyrosine) in the active site are shown for orientation purposes. [ConSurf (http://consurf.tau.ac.il/); Glaser et al. 2003]

When developing a drug that targets an individual member of a larger protein family, it is useful to have a complete overview of all proteins in that family to avoid unintentional development of compounds that bind to nontarget family members. With access to the almost finished version of the public human genome sequence (Build 33), the composition of the PTP family was recently defined. A total of 38 human classical PTP genes and 12 pseudogenes were identified, just adding one novel gene to the previously defined non-redundant database of vertebrate PTP transcripts (Andersen et al. 2001; see http://science.novonordisk.com/ptp and http://ptp.cshl.edu for a detailed analysis of the PTP family).

4 Structural Requirements for Substrate Specificity

In addition to information on the catalytic machinery, the first X-ray structures of PTP1B demonstrated a common binding mode of pTyr substrates: (1) binding of the phosphate moiety of pTyr to the P-loop (residues 214– 222) and Arg221, (2) hydrophobic interactions of the phenyl ring of pTyr with the active site pocket and (3) hydrogen bonds between the main chain nitrogens of the pTyr substrate (pTyr and residue +1) and the side chain of Asp48 of PTP1B, and (4) a hydrogen bond between the main chain carbonyl of residue -2 and the main chain nitrogen of residue 47 (Jia et al. 1995; Sarmiento et al. 1998). Specificity is provided by side chain interactions of the peptide and PTP1B (and other PTPs) (Yang et al. 2000), which has served as inspiration in early approaches to structure-based design of PTP inhibitors (see below).

A number of enzyme kinetic analyses with synthetic substrates and peptide-based inhibitors have provided insight into the fine specificity of PTPs (e.g., Cho et al. 1993; Ruzzene et al. 1993; Zhang et al. 1993; Zhang et al. 1994a; Desmarais et al. 1998; Desmarais et al. 1999). In an early and elegant study, Dixon and coworkers found that a catalytically inactive [³⁵S]-labeled PTP1B_{C2155} mutant, i.e., the first 'trapping mutant' (see below, Sect. 5), bound to activated, tyrosine phosphorylated epidermal growth factor receptor (EGF-R) with high affinity (K_d =100 nM) (Milarski et al. 1993). Synthetic tyrosine phosphorylated peptides corresponding to the four major autophosphorylation sites in the EGF-R were tested for their ability to displace PTP1B_{C2155} from the full-length EGF-R. Although PTP1B was displaced by all four peptides, the peptides containing pTyr¹¹⁴⁸ or pTyr⁹⁹² were the most potent. Peptides and peptide analogs corresponding to the EGF-R sequence around residue 992 (i.e., D-A-D-E-pY-L-I-P-Q-Q-G with pY=pTyr) have subsequently served as extremely useful tools in enzyme kinetic and structural analyses of PTP substrate specificity, and in inhibitor design, as will be discussed in the following. In a detailed analysis of the size and pTyr positioning requirements, it was found that efficient binding and catalysis was provided by Ac-D-A-D-E-pY-L-NH₂ (Zhang et al. 1994a). A structural explanation for efficient binding of substrates with acidic residues immediately N-terminal to the pTyr residue was provided by the first X-ray structures of PTP1B in complex with this EGF-R-derived peptide, demonstrating specific interactions between the acidic residues and Arg47 of PTP1B (Jia et al. 1995; Sarmiento et al. 1998). Additional support for this notion was obtained by molecular modeling of D-A-D-E-X-L-based inhibitors in CD45 and LAR (Glover and Tracey 2000).

Using 'inverse alanine scanning,' Zhang and coworkers further assessed the substrate specificity of PTP1B (Vetter et al. 2000). Separate and sequential replacement of each alanine residue in the parent peptide, Ac-AAAApYAAAA-NH₂, with the 19 other natural amino acids, identified Ac-ELEFpYMDYE-NH₂ as a highly potent PTP1B substrate. As found previously, a strong preference was observed for acidic residues. Additionally, it was noted that PTP1B could also accommodate hydrophobic and aromatic residues at the -1 position. The structural basis for this plasticity was revealed by X-ray crystallographic studies showing that accommodation of both acidic and hydrophobic residues in the -1 position was conferred by Arg47, which can adopt different conformations, thereby generating two sets of distinct binding modes (Sarmiento et al. 2000).

5 Identification of Natural Substrates: The Insulin Receptor-Regulating PTP

The above studies with synthetic peptides have advanced our understanding of the substrate specificity of PTP domains. However, in vivo PTPs act in the context of full-length folded proteins, and hence there is a need for gaining insight into the three-dimensional structural requirements for substrate specificity. Based on the understanding of the catalytic machinery (Sect. 2), Tonks and coworkers have devised an intriguing strategy by creating socalled trapping mutants, i.e., PTP mutants that are inactive or display very low activity yet retain their 3D structure intact, as well as most of the functional features needed for substrate binding and catalysis, including closure of the WPD loop (Flint et al. 1997). In particular, PTPs which are either mutated at position 215 (Cys to Ser) or at position 181 (Asp to Ala) have been used widely and have provided significant information on PTP substrate specificity in intact cells. Recently, and again based on an understanding of PTP-mediated catalysis at the molecular level, the affinity of the Asp181Ala mutant was increased considerably by introducing an alanine in position 262, i.e., the invariant glutamine that positions the nucleophilic water molecule in the second step of hydrolysis (Sect. 2). It is anticipated that this mutant, due to its higher affinity for pTyr peptides, can be used to isolate lessabundant PTP substrates (Xie et al. 2002).

The definition of the exact function of a protein requires inter- and multi-disciplinary approaches and is often based on stepwise investigations over the years. A number of approaches have been used to assist the identification of the function of specific PTPs. These studies have been reviewed in detail elsewhere, and only one example will be given to illustrate the importance of modern structural biology and the relevance of creating an intimate connection between basic science and drug discovery.

In a landmark publication, Tremblay, Kennedy, and colleagues reported that PTP1B knockout mice display increased insulin sensitivity and resistance to diet-induced obesity (Elchebly et al. 1999), a finding that was later confirmed in an independent study (Klaman et al. 2000). In the present context, it is of relevance that these studies pointed to a direct negative regulatory effect of PTP1B on the insulin receptor tyrosine kinase (IRTK). To provide a framework for the rational development of specific inhibitors of this interaction, Barford and Tonks embarked on a detailed analysis by integrating X-ray crystallography, enzyme kinetics, and peptide-binding studies, and provided strong evidence for simultaneous binding of tandem pTvr residues in the IRTK activation loop to the active site and an adjacent pTyr recognition site (Salmeen et al. 2000). The latter site, the second aryl phosphate binding site (site 2), had previously been proposed to be used for the design of specific and potent inhibitors of PTP1B (Puius et al. 1997). Further, to bind simultaneously to the active site and site 2, the substrate has to pass through (or fit in) a cleft-like part of PTP1B, which we have used in our design of selective PTP1B inhibitors (Peters et al. 2000; Iversen et al. 2001) (Sect. 9.2.5). Tonks and coworkers next used bioinformatic analyses to identify other protein kinases with tandem pTyr residues in the activation loop as potential substrates for PTP1B, and in fact evidence was provided for the notion that TYK2 and JAK2 are substrates of PTP1B (Myers et al. 2001). This is of particular interest since this might clarify part of the phenotype of the PTP1B knockout mice, i.e., resistance to diet-induced obesity, which is not easily explained by an effect of PTP1B on the IRTK-mediated anabolic signaling. Rather, such a phenotype could be due to the absence of a negative regulatory effect of PTP1B on the leptin system, which signals via JAK2. Subsequent studies of the two strains of PTP1B knockout mice provided evidence that indeed leptin signaling is increased in these mice (Cheng et al. 2002; Zabolotny et al. 2002). Together, these studies not only offer a solid biological basis for identification of PTP1B as an important drug target in diabetes and obesity, which was recently strongly supported by antisense studies in vivo (Zinker et al. 2002), but also structural inspiration for design of compounds that specifically inhibit PTP1B.

6 Nature's Regulation of PTP Activity

Aberrant tyrosine phosphorylation levels have been linked both to cancer and metabolic disease states. Hence, tight regulation and specificity of PTPs is required. Nature may achieve control of PTP activity by a number of different mechanisms, as exemplified in the following: (1) Site-specific tyrosine phosphorylation and dephosphorylation may result from inherent substrate specificity of kinases and phosphatases. (2) The interaction between PTPs and their cognate substrates may be regulated by subcellular localization which can be controlled either by targeting domains of the PTPs ('zip codes') (Mauro and Dixon 1994), alternative splicing (Shifrin and Neel 1993), or proteolytic cleavage (Frangione et al. 1993; Gil-Henn et al. 2000, 2001; Ragab et al. 2003). (3) Different isoforms of PTPs may be synthesized from the same gene due to alternative usage of isoform-specific 5' exons and promoters (Elson and Leder 1995; Tanuma et al. 1999; Amoui et al. 2003). (4) The enzymatic activity of PTPs may further be influenced by covalent modification such as phosphorylation of specific tyrosine and serine/threonine residues (Flint et al. 1993; Moeslein et al. 1999; Ravichandran et al. 2001), or by dimerization (Bilwes et al. 1996; Majeti et al. 1998; Jiang et al. 1999; Blanchetot and Den Hertog 2000; Jiang et al. 2000; Majeti et al. 2000; Blanchetot et al. 2002a), or by oxidation of the active site cysteine (see below). (5) As with other enzymes, the cellular levels of PTPs may be further controlled by specific transcription factors (Fukada and Tonks 2003), or by the half-life of the mRNA or protein of the PTP in question.

Although most of the above principles for regulation of PTP activity and specificity could potentially be utilized for development of drugs that modulate the activity of PTPs, the most straightforward approach—and also the focus of most laboratories—is to develop active site-directed inhibitors. It should be emphasized that covalent modification of PTPs on residues in the proximity of the active site, e.g., phosphorylation of Ser50 in PTP1B (Ravichandran et al. 2001), might potentially influence the activity of inhibitors that are developed against the non-modified enzyme. It is therefore an inherent risk in a drug optimization program to rely exclusively on recombinant, non-modified proteins expressed in prokaryotic systems.

Recombinant PTPs show relatively high catalytic activity compared to protein tyrosine kinases (Denu and Dixon 1998). Therefore, it appears that there is a need for transient, reversible inactivation of PTPs to ensure proper cellular tyrosine phosphorylation levels and thereby efficient propagation of signaling. In the present context, it is of particular relevance that nature seems to regulate PTP activity by H_2O_2 -mediated reversible oxidation of the active site cysteine. It took about three decades from the initial biological observations to unravel the structural basis for the effects of H_2O_2 on PTPs. In the early 1970s, Michael Czech and his colleagues made the intriguing observation that thiols mimic the action of insulin on isolated fat cells (stimulating glucose uptake and metabolism and inhibiting lipolysis) by Cu^{2+} -dependent production of H_2O_2 (Czech et al. 1974a,c). Further, it was shown that extracellular addition of H_2O_2 can mimic the action of insulin in fat cells (Czech et al. 1974c), and evidence was provided for involvement of sulfhydryl oxidation in this process (Czech et al. 1974b). The physiological relevance of these findings became apparent when it was demonstrated that insulin stimulates intracellular formation of H_2O_2 in rat epididymal fat cells (May and de Haen 1979).

A large number of other agents have also been shown to mimic the action of insulin in isolated cells, including vanadate (Dubyak and Kleinzeller 1980), which was shown to act synergistically with H₂O₂ to stimulate the insulin receptor kinase activity in intact cells in an apparently direct manner (Tamura et al. 1984; Kadota et al. 1987; Koshio et al. 1988; Heffetz et al. 1990; Heffetz and Zick 1992). However, it was not realized until 1989 that the synergistic effects of H₂O₂ and vanadate were due to the formation of "pervanadate", which increases the phosphorylation and activity of the IRTK, not directly, but by inhibiting counteracting PTP(s) (Fantus et al. 1989). This seminal publication was the first to demonstrate that perhaps it would be possible to mimic the action of insulin by inhibiting PTP(s) that negatively regulate the insulin signaling pathway. Indeed, this publication played a significant role for initiation of the PTP inhibitor drug discovery program at Novo Nordisk. In the present context, it is intriguing that recent studies have demonstrated that insulin-stimulated formation of H₂O₂ leads to reversible inhibition of PTP1B and enhancement of the insulin signaling in 3T3-L1 cells (Mahadev et al. 2001a,b).

In 1998, it was demonstrated at the biochemical level that PTPs could be negatively regulated by H₂O₂ (Denu and Tanner 1998). PTPs react rapidly with low micromolar concentrations of H₂O₂, specifically oxidizing the active site cysteine and involving a sulfenic acid intermediate. Importantly, the inactivation took place in the presence of reducing agents and it was reversible, thus providing support for the view that PTP function may be regulated in vivo by reactive oxygen species. In an attempt to determine the structure of PTP1B with the catalytic cysteine oxidized to the sulfenic acid state, Barford, Tonks, and colleagues unexpectedly observed a modification of the active site cysteine, termed "sulphenyl-amide", which was indicated by a welldefined continuous electron density between the Sy atom of Cys215 and the main chain nitrogen atom of Ser216 in the X-ray structure (Salmeen et al. 2003). As a result, the P-loop undergoes a major conformational change (with Gly218 moving about 7 Å outwards) and the oxidized cysteine is now exposed to the reducing environment in the cell, thus facilitating the regeneration of the active state of the enzyme. Moreover, the conformational change imposed by the sulphenyl-amide bond formation results in disruption of the hydrogen bond between Ser216 and Tyr46 in the pTyr loop, and

the latter tyrosine residue adopts a solvent-exposed position. Interestingly, this repositioning of Tyr46 makes PTP1B susceptible to phosphorylation by the insulin receptor, and the authors propose that such redox-dependent phosphorylation may represent an additional level of control over the signaling specificity of PTP1B. These structural studies together with the above biochemical studies provide not only a plausible novel mechanism for PTP regulation, but also a unique possibility for designing compounds that only influence signal transduction pathways that are already activated (see Sect. 10). Of note, the structural changes of PTP1B observed by addition of H₂O₂ may also be induced by addition of putative inhibitors of the enzyme (van Montfort et al. 2003; unpublished observations).

Interestingly, UV irradiation causes inactivation of PTPs and converts PTP α into a substrate-trapping mutant which can coprecipitate the plateletderived growth factor (PDGF)- β receptor, similarly to the PTP α_{C433S} mutant (Gross et al. 1999). Further, den Hertog et al. have recently shown that H₂O₂ treatment of cells leads to rotational coupling and inactivation of PTP α dimers (Blanchetot et al. 2002b; van der Wijk et al. 2003).

Using a modified 'in-gel' PTP assay to allow visualization of oxidized PTPs, Tonks and coworkers recently provided evidence for the notion that reversible oxidation may be a general mechanism for regulation of PTP activity (Meng et al. 2002). Specifically, these authors showed that ligand-induced, transient oxidation of SHP-2 in the PDGF-R complex coincided with autophosphorylation of the receptor, thus demonstrating that PTPs not only respond to oxidative stress in the environment, but also to reactive oxygen species generated to physiological responses, as in the case of insulin signaling.

7 PTPs as Drug Targets

In the early days of PTP research, these enzymes were mainly considered as housekeeping enzymes that turned signaling processes off. A more complicated picture later emerged showing PTPs both to be negative and positive regulators of signaling pathways. Despite this, most drug discovery activities in the PTP field have so far been directed towards development of inhibitors of PTPs that negatively regulate signaling pathways, thereby intensifying and/or prolonging signaling.

In particular, the insulin signaling pathway has received much attention based on the assumption that the insulin-resistant state in type 2 diabetes could be overcome by inhibiting PTPs that negatively regulate this pathway. Based on theoretical considerations, our own efforts began back in the early 1990s by re-cloning and expression of PTP1B as a tool (Hoppe et al. 1994), searching for novel PTPs (Møller et al. 1994a,b), and by studying the tissue distribution of PTPs (Norris et al. 1997). We decided to focus on PTPs that negatively regulate upstream elements in the insulin-signaling pathway, preferentially the insulin receptor tyrosine kinase itself (Møller et al. 1995). We assumed that even the unstimulated IR had to be controlled by counteracting PTPs, i.e., potentially selective inhibitors could be used not only to treat patients with type 2 diabetes, but also type 1 diabetes. Numerous studies have since then been devoted to identification of the insulin receptor-regulating PTPs (IR-PTP) and the development of selective inhibitors thereof (reviewed in Kennedy 1999; Møller et al. 2000; Ramachandran and Kennedy 2003; Zhang and Lee 2003), namely: (1) brute force substrate trapping studies (Walchli et al. 2000); (2) in vitro studies with anti-sense oligonucleotides (Kulas et al. 1995); (3) tissue distribution analyses (Norris et al. 1997); (4) studies of the dynamics in subcellular distribution of PTPs (Calera et al. 2000); (5) insulin-based selection system analyses (Møller et al. 1995); (6) overexpression studies. As a result, several candidate PTPs have been named, including PTP1B, PTP α (Møller et al. 1995; Cong et al. 1999), and PTP-LAR (Ahmad et al. 1995; Kulas et al. 1995). As described above, PTP1B is clearly the PTP target that has been validated and addressed most in the diabetes and obesity field.

As recently pointed out by one of the key founders of the PTP field, it is important to remember that there is more to the PTP family than just PTP1B (Tonks 2003), and it should be remembered that other PTPs, in addition to PTP1B, may be involved in the negative regulation of insulin signaling. Also, other PTPs could potentially be attractive drug targets, such as CD45 in autoimmunity (Hermiston et al. 2003; Irie-Sasaki et al. 2003; Lee and Burke 2003) and SHP-1 in cancer (Wu et al. 2003). Further, other PTPs seem to be negative regulators of the immune system, by recruitment to receptor molecules with ITIMs (immunoreceptor tyrosine inhibitory motifs). It might therefore be speculated that inhibitors of such PTPs (e.g., SHP-1) enhance the activity of cells like natural killer cells (Jackson 2003). Additionally, LAR (Mooney and LeVea 2003) and PTP α (Pallen 2003) have been proposed as potential drug targets, and SHP-2 mutants are critically involved in the development of Noonan syndrome and juvenile myelomonocytic leukemia (Tartaglia et al. 2003).

In addition, PTP inhibitors may be useful for treatment of infectious diseases (reviewed in van Huijsduijnen et al. 2002). Thus, it has been found that *Helicobacter pylori*, a class I carcinogen, produces a protein, CagA, that activates SHP-2 leading to a growth factor-like response in gastric epithelial cells (Higashi et al. 2002; Hatakeyama 2003). Also, PTP ζ and PTP α appear to be receptors for the *H. pylori* cytotoxin, VacA, that induces vacuolation, mitochondrial damage, cytochrome *c* release, and apoptosis of gastric epithelial cells (Yahiro et al. 1999; Yahiro et al. 2003). A number of bacterial pathogens have evolved strategies to destabilize signal transduction for their own benefit, and highly active PTPs seem to be used as such sophisticated virulence factors (Clemens et al. 1991). As an example, the *Yersinia* PTP, which has served as an excellent research tool in a number of publications, has been shown to be essential for virulence (Guan and Dixon 1990). Similarly, a PTP in *Salmonella* seems to be required for full display of virulence (Kaniga et al. 1996; Murli et al. 2001). Ullrich and coworkers recently cloned and characterized two secretory PTPs from *Mycobacterium*, one of which was proposed as a candidate virulence gene (Koul et al. 2000).

It remains to be shown if some or all of the above-mentioned PTPs will eventually prove to be good drug target candidates. Clearly, additional basic science and preclinical work will be required to validate these candidates. In any case, selective, cellularly active PTP inhibitors would be invaluable tools in such assessments.

8 Reversible, Competitive Inhibitors

8.1 Challenges

Initially, the pharmaceutical industry was reluctant to enter the PTP inhibitor field, and in particular to developing active site-directed inhibitors. The highly conserved structure of the catalytic domains of even distantly related PTPs with little primary sequence identity in combination with the expectation of a large protein family (Charbonneau and Tonks 1992; Pot and Dixon 1992a; Denu and Dixon 1998) seemed to represent insurmountable obstacles for the development of highly specific, low molecular weight inhibitors for therapeutic use (reviewed in Tonks 2003). This was also the situation in the early days in the kinase field (Cohen 2002). In the latter case, the development of a relatively specific and clinically extremely efficient kinase inhibitor, Gleevec, for the treatment of chronic myelogenous leukemia and other malignant diseases has changed the perception completely in the industry (see chapters by Cohen and Wakeling, this volume).

In our opinion, the major challenge in developing PTP inhibitors is not related to the conserved structure of PTPs as such, but rather to the very nature of the PTP catalytic machinery (Sect. 2). Indeed, we and others have demonstrated that highly selective and potent PTP inhibitors can be made (see Sect. 9). However, while the above-described low pK_a value of the catalytic cysteine in PTPs is essential for its function as a nucleophile both in the first step of catalysis and for the susceptibility to redox regulation, this same property is one of the major challenges in the identification and development of PTP inhibitors. Thus, it is a common experience among laboratories that perform high-throughput screenings to get high hit rates when searching for PTP inhibitors, in many cases caused by oxidation or alkylation of the active site cysteine (Bleasdale et al. 2001; van Montfort et al. 2003) (see below). It should be noted, however, that redox-induced conformational changes of PTPs as discussed above (Sect. 6) may represent a novel opportunity for the design of inhibitors that only influence already activated signaling pathways (Sect. 10).

Another challenge relates to the nature of the pocket of the active site of PTPs. In contrast to kinases, for which inhibitors have been developed that bind to a relatively hydrophobic pocket in the ATP binding site, the active site in PTPs is a water-molecule-filled, fairly shallow, cavity that first turns into a pocket upon closure of the WPD loop (Sect. 2). A prerequisite for this loop closure seems to be binding of the phosphate moiety of pTyr to the P-loop. Thus, many structure-based approaches are based on development of inhibitors that mimic the binding of phosphate to the P-loop. Since pTyr at neutral pH is doubly negatively charged, most competitive PTP inhibitors developed to date are similarly charged molecules. As a consequence, problems with cellular uptake, pharmacokinetic properties, and oral availability may be expected.

8.2 Identification of Chemical Lead Structures

To identify small molecule PTP lead structures, the most straightforward method is to mimic the natural substrate pTyr by making non-hydrolyzable analogs. Initial attempts in academia were very successful in developing model compounds as described below (Sect. 9.1). Since these compounds often showed poor or no cellular activity, most companies turned to high-throughput screening (HTS) in attempts to identify novel lead structures. At Novo Nordisk we used a scintillation proximity assay (SPA) with PTP1B and a biotinylated [³³P]-radiolabeled peptide substrate corresponding to the activation loop of the insulin receptor tyrosine kinase. A substantial number of hits were identified in the screening. However, when analyzing these hit compounds in detail, as recommended below, most were eliminated as false positives. The compound that fulfilled most of our pre-defined selection criteria (see below) was 2-(oxalylamino)-benzoic acid, OBA (compound 1, Fig. 4; Andersen et al. 2000). This compound was found to share most of the

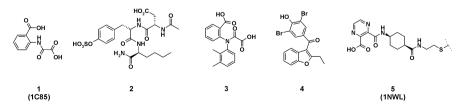


Fig. 4 Chemical lead structures identified by different strategies

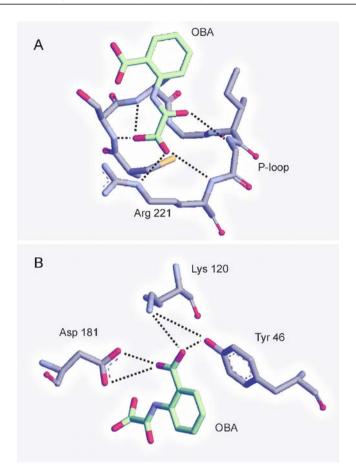


Fig. 5A, B Binding mode of OBA in PTP1B. A Interaction with the P-loop. B Unique interaction with Lys120, Asp181, and Tyr46

interaction points with the natural substrate, pTyr, and in addition showed a unique arrangement involving residues 46, 120, and 181 (pdb code 1C85– Fig. 5A and B). As will be detailed below, this simple compound has served as a basic scaffold in our development of highly potent and selective PTP inhibitors. Also, other companies have employed HTS to identify chemical leads. Initially, Bleasdale and coworkers used a broad screening strategy. However, their search for reversible, competitive inhibitors was confounded by identification of many compounds representing several chemical classes that irreversibly inhibited PTP1B by reacting with Cys215 (Bleasdale et al. 2001). Using targeted screening of a collection of compounds with suspected or known pTyr bioisosteres, these authors identified a series of relatively potent PTP1B inhibitors based on sulfotyrosyl-containing CCK-8 derived octapeptides. The common denominator of these peptides was an N-terminal tripeptide, which inhibits PTP1B with a K_i value of 5 μ M (compound 2, Fig. 4; Bleasdale et al. 2001), which was in turn used for lead optimization.

To avoid the problem with interference from oxidizing and alkylating agents, two groups have developed SPA-based screening assays using PTP1B_{C215S} mutants (Skorey et al. 2001; Wang et al. 2001). Although this seems to be an attractive approach, it should be noted that there is a considerable risk that low-potency compounds will be missed in a screen using this mutant. Hence, both thermodynamic and X-ray crystallographic studies have recently shown that the P-loop in the PTP1B_{C215S} mutant is in a different conformation compared to the wild-type enzyme (Zhang et al. 2000; Scapin et al. 2001). Binding of a pTyr peptide can stabilize the binding of the P-loop in the PTP1B_{C215S} mutant to obtain the wild-type conformation (Barford et al. 1994; Jia et al. 1995), but this cannot be expected with compounds that only utilize some of the interaction points with the P-loop. Indeed, we have recently shown that some analogs of OBA do not bind efficiently to the PTP1B_{C215S} mutant (unpublished results, N.P.H. Møller et al.).

Liu, Jirousek, and their colleagues at Abbott employed a nuclear magnetic resonance (NMR)-based screen with uniformly [¹⁵N]-labeled PTP1B to identify a chemical PTP1B inhibitor lead structure (Liu et al. 2003a). The screening of more than 10,000 compounds lead to the identification of 2,3-dimethylphenyloxalylaminobenzoic acid (compound 3, Fig. 4) as an active site binder with a K_i value of ~90 μ M, thus again demonstrating the usefulness of oxamic acid-based compounds as phosphate bioisosteres. In contrast to OBA, analogs of compound 3 bind to PTP1B in the open conformation (Liu et al. 2003a).

In another PTP1B inhibitor-development program, early emphasis was given to orally availability and pharmacokinetic properties (Wrobel et al. 1999). A potent uricosuric agent benzbromarone (compound 4, Fig. 4) that showed weak inhibition of rat PTP1B was used as a starting point for lead optimization, resulting in compounds which showed glucose lowering properties in *ob/ob* mice. One of these compounds has recently been withdrawn from phase II clinical trials, and it has been reported that the glucose-lowering effects may be attributed to PPAR- γ -activating activity of the compound (reviewed by Johnson et al. 2002).

Recently, an intriguing approach to discover novel pTyr mimetics for PTP1B was described using the 'breakaway tethering' approach (Erlanson et al. 2003). Since the active site cysteine prevents using this approach directly, a novel strategy was designed. Arg47 was mutated to a cysteine residue and labeled with an 'extender' that included an oxalic acid to protect Cys215 against alkylation. Further, an oxalic acid-based thiol was used as positive control, again demonstrating the usefulness of this moiety in the discovery of PTP inhibitors. Tether screening of 15,000 disulfide-containing fragments identified compound 5 (Fig. 4) as one of the strongest hits. Importantly, the

free compound 5 displayed competitive inhibition of PTP1B, although with low potency ($K_i \sim 4$ mM). The X-ray crystal structure of compound 5 disulfide-bonded to the extender-modified PTP1B_{R47C} showed several hydrogen bonds from the carboxylic acid and pyrazine moieties to the active site, and the cyclohexyl ring fits well in the hydrophobic pocket normally occupied by the phenyl ring of pTyr (pdb code 1NWL).

8.3 Recommendations and Guidelines

Our first HTS efforts at Novo Nordisk in 1996 was aimed at the identification of a general PTP inhibitor scaffold, and the number of verified hits far exceeded our initial expectations. As described above, in these screens we used a scintillation proximity assay with PTP1B and a [³³P]-radiolabeled peptide substrate. In these early days, the PTP(s) that negatively regulate the IR signaling pathway had not yet been identified, although several candidates had been proposed including PTP1B, LAR, and PTP α (see above). Hence, our approach was to identify a general, low molecular weight inhibitor that could be optimized for specificity against a number of different PTPs (Møller et al. 2000) (Fig. 6). These inhibitors should then in turn be used in cell-based assays to identify the PTP that negatively regulates the IR. As soon as a hit had been resynthesized and confirmed, we initiated further validation of the hit

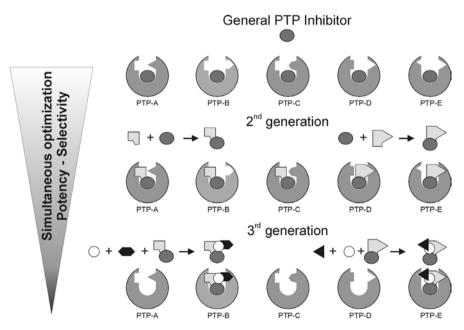


Fig. 6 Schematic representation of the Novo Nordisk lead optimization strategy

by synthesizing a number of analogs and by testing these for selectivity in biochemical assays (against other PTPs) and cell-based assays.

A variety of chemical classes were identified and several promising lead series were pursued. In particular, much attention was given to a series that included compounds that significantly induced insulin-independent glucose uptake in isolated rat soleus muscle. However, despite the synthesis of a large number of analogs within these lead series, we did not succeed in obtaining significant improvement of potency or selectivity. Indeed, rarely did we find a true structure-activity relationship (SAR). Further in-depth analyses revealed that many of these compounds showed mixed-type inhibition and, perhaps most significantly, the inhibition of PTPs depended strongly on the assay conditions, most likely because the apparent inhibition was caused by oxidation or alkylation of Cys215: (1) In some cases inhibition was abolished by using dithiothreitol (DTT at 1 mM), rather than reduced glutathione (GSH at 5 mM) as reducing agent in the assay buffer (i.e., the observed inhibition most likely was due to oxidation of the catalytic cysteine, which was prevented by the stronger reducing agent DTT). (2) On the contrary, other compounds only displayed PTP inhibitory activity in the presence of DTT, and this inhibition could be prevented by addition of ethylenediaminetetraacetate (EDTA). This type of compound most likely inhibits PTPs by a mechanism that resembles that reported for alendronate, i.e., formation of H₂O₂ by a combination of the compounds with low micromolar concentrations of DTT and trace metals (present in the buffer) (Skorey et al. 1997). In such cases, the addition of catalase eliminated the inhibition. It is likely that several compounds reported in the literature could represent false positive hits (Bleasdale et al. 2001; van Montfort et al. 2003). Further, the inhibition of some compounds was reduced significantly or eliminated by addition of carrier protein such as albumin. This observation could be attributed to high affinity binding to albumin. However, the inhibition of yet other compounds was completely abolished by addition of albumin without any stoichiometric relationship. To avoid false-positive compounds we designed a straightforward and simple test routine as detailed below.

Since the purpose of the HTS was to identify compounds that were general and classical competitive inhibitors, we defined the following selection criteria for positive hits. The compounds should show (1) reversible, (2) competitive, (3) time-independent, and (4) assay-independent inhibition of more than 3 out of 4 different PTPs (Fig. 6).

Based on our experience, we recommend evaluation of potential hits by a practical scheme similar to that depicted in Fig. 7. The first round of analyses is performed in parallel in 96-well plate format. Robust compounds should be identified by testing for type of inhibition in different assay systems. We recommend using at least two reducing systems: (1) GSH with addition of EDTA to chelate trace metal ions and (2) DTT. Further, albumin or a detergent should be added. Compounds must show similar K_i values and

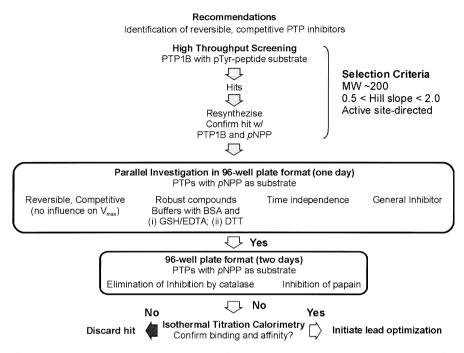


Fig. 7 Recommendations: Identification of reversible, competitive chemical leads for structure-based design of selective PTP inhibitors

competitive type of inhibition in both buffer systems (i.e., no influence of V_{max} app with increasing inhibitor concentration), and they should not be time dependent. In our case, we further require that lead compounds are general PTP inhibitors and consequently test a small battery of different PTPs. In the next round, we analyze for sensitivity to catalase (which would be an exclusion criteria) and include a specificity test by testing for activity against non-PTP enzymes with a catalytic cysteine such as papain (Fig. 8).

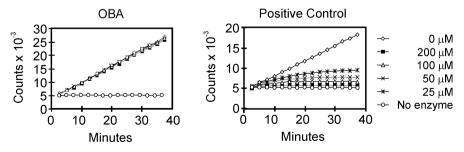


Fig. 8 Specificity check of putative PTP inhibitors in papain. OBA showing no inhibition and a non-specific PTP inhibitor demonstrating time-dependent inhibition of papain

Recently, we have as a final test included isothermal titration calorimetry to verify that the compounds indeed bind to the PTPs.

The power of our approach is probably best illustrated by the fact that OBA was selected as a potential lead structure exclusively on the basis of its behavior as a classical, textbook example of a reversible, competitive inhibitor. Indeed, the label on the vial containing the original HTS hit indicated a different structure, and it was not until after structural analysis that the true nature/structure of OBA was revealed.

In addition to the above PTP-specific problems, it appears that there is a general problem related to screening of compound libraries. Thus, further analysis of screening hits often reveals peculiar inhibition properties showing non-competitive binding with poor specificity and little relationship between structure and activity. In a careful analysis of 'general screening hits' it was found that the inhibition of such compounds could largely be eliminated by increasing the concentration of the enzymes by 10-fold despite a 1,000-fold excess of inhibitor (McGovern et al. 2002). Further analysis revealed that such compounds formed aggregates, and it was suggested this may account for the activity of many promiscuous screening hits (McGovern et al. 2002; McGovern and Shoichet 2003). It is advisable to add this simple procedure to our scheme depicted in Fig. 7.

9 Structure-Based Design—PTP1B Inhibitors

PTP1B, the first PTP to be isolated and characterized, has been leading the way in the development of PTP inhibitors (Tonks et al. 1988a, 1988b). This is partly because early studies indicated a role for PTP1B in the negative regulation of insulin signaling and hence its potential as drug target in diabetes (Sect. 7). However, the key determining factor seems to be that PTP1B and its rat ortholog, PTP1, have been used in numerous studies as model enzymes to investigate the mechanism of action of PTPs and the structural basis for substrate specificity. As early as 1994, the crystal structure of PTP1B was reported (Barford et al. 1994), and this was quickly followed by a seminal publication describing the structural basis for phosphotyrosine peptide recognition by PTP1B (Jia et al. 1995). Currently, about 40 different PTP1B X-ray structures have been deposited in the public domain (see Table 1).

Based on the above structural information, academia spearheaded the search for selective PTP inhibitors by development of non-hydrolyzable peptide mimetics (reviewed in Burke and Zhang 1998).

9.1 Peptide-Based Inhibitors—Phosphotyrosyl Mimetics

The initial attempts to design selective PTP inhibitors were based on replacement of pTyr with non-hydrolyzable phosphotyrosyl mimetics in small, efficient PTP peptide substrates, thereby utilizing both the potency and selectivity provided by the amino acid residues (reviewed in Burke and Lee 2003). The next step in inhibitor design was then planned to be modification of the peptide moiety into more drug-like compounds without losing potency or selectivity. The most straightforward solution was to replace the phosphate moiety with phosphonates, i.e., by replacing the scissile oxygen in pTyr with a non-hydrolyzable chemical group such as methylene, resulting in (phosphonomethyl)-phenylalanine (Pmp) (Domchek et al. 1992; Smyth et al. 1992). When Pmp is replacing pTyr in the Ac-D-A-D-E-pY-L-NH₂ peptide, a relatively potent inhibitor of PTP1B is obtained (i.e., $K_i \sim 10 \ \mu M$ —compound 6, X=CH₂, Fig. 9) (Zhang et al. 1994a). Introduction of two fluorines at the α -methylene position in Pmp yielded F₂Pmp. Interestingly, the peptide Ac-D-A-D-E-F₂Pmp-L-NH₂ (compound 7, Fig. 9) showed ~1,000-fold increase in affinity for the rat PTP1 compared with the Pmp peptide (Burke et al. 1994; Chen et al. 1995). This dramatic increase in affinity was due to direct interaction of the fluorines with the enzyme, rather than changes in pK_a values, as revealed at the structural level by co-crystallizing PTP1B with the non-peptidyl inhibitor [1,1-difluoro-1-1(2-naphthalenyl)-methyl]phosphonic acid (compound 18, Fig. 11) (Burke et al. 1996b) (see below). Subsequently, it

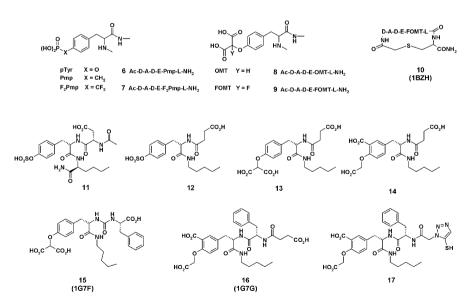


Fig. 9 Phosphotyrosyl mimetics—peptide-based inhibitors

was found that two F₂Pmp moieties in a sequence-specific manner could be used to improve the potency and selectivity of tripeptides (Desmarais et al. 1999). Thus, the tripeptide, Glu-F₂Pmp-F₂Pmp inhibited PTP1B with an IC₅₀ of 40 nM, which represented more than 100-fold selectivity against CD45, PTP β , and LAR. Interestingly, Pro-F₂Pmp-F₂Pmp is equipotent against PTP1B and PTP β , with IC₅₀ values in the 0.2–0.3 μ M range.

To improve bioavailability, efforts have been directed towards the development of less-charged, non-phosphorus-containing, peptide inhibitors. One of the first steps away from peptidyl phosphonate-based inhibitors was taken by Burke and colleagues. Based on previous work (Miller et al. 1994), L-O-malonyltyrosine (OMT) was introduced as a relatively efficient pTyr analog in synthetic peptides used to inhibit SH2 domain interactions (Ye and Burke 1995; Ye et al. 1995). As in the above studies with F₂Pmp, OMT was introduced in parallel into the EGF receptor peptide yielding Ac-D-A-D-E-(OMT)-L-NH₂ (compound 8, Fig. 9) that showed about a 20-fold increase in potency against PTP1B compared with Pmp (Kole et al. 1995a). Based on the experience with FPmp and F₂Pmp, these authors next incorporated fluorine in OMT yielding 4'-O-[2-(2-fluoromalonyl)]-l-tyrosine, FOMT, and synthesized Ac-D-A-D-E-(FOMT)-l-NH₂ (compound 9, Fig. 9), which showed a tenfold increase in affinity for PTP1B ($K_i \sim 1 \mu M$) compared with the unfluorinated counterpart ($K_i \sim 13 \mu M$) (Burke et al. 1996a). When restraining the conformational flexibility of these peptides, a substantial increase in affinity for PTP1B was observed with a K_i of 0.7 μ M for a cyclic OMT-based peptide (Akamatsu et al. 1997) and a K_i of 0.17 μ M for the cyclic FOMT-peptide (compound 10, Fig. 9) (Akamatsu et al. 1997).

With the above relatively potent PTP1B inhibitors at hand, Barford, Burke, and their colleagues next examined the structural basis for the inhibitory capacity of these compounds (Groves et al. 1998). The cyclic FOMT-peptide was found to bind to PTP1B with the WPD loop in the open conformation (pdb code 1BZH). This is probably due to the fact that FOMT is less efficient than pTyr in forming hydrogen bonds with the P-loop and the side chain of Arg221 and further, the aromatic ring of the FOMT peptide is displaced 1.4 Å away from the P-loop compared to natural substrate, thus not obtaining stabilization of the closed conformation of the WPD loop via π - π interaction with Phe182 as seen in the PTP1B–pTyr peptide complex (Jia et al. 1995).

The baton from the above studies was later picked up at Pharmacia, where it was serendipitously discovered that peptide analogs of cholecystokinin (CKK-8) were relatively potent inhibitors of PTP1B with a common N-terminal tripeptide, N-Ac-D-Tyr(SO₃H)-Nle-, being necessary and sufficient for inhibition (Bleasdale et al. 2001). N-Ac-D-Tyr(SO₃H)-Nle-NH₂ (compound 11, Fig. 9) was found to inhibit PTP1B with a K_i value of 17 μ M. Importantly, removal of the two terminal moieties resulted in a compound with only a twofold reduction in affinity (compound 12— K_i =30 μ M). Previously, a synthetic tris-sulfotyrosyl dodecapeptide analog of the insulin-receptor 1146-kinase domain was shown to inhibit dephosphorylation of the insulin-receptor (Liotta et al. 1994; Kole et al. 1996). Replacing the tyrosinesulfate in the above N-terminal CCK-derived peptide with either OMT (13) (Kole et al. 1995a) or 2-carboxymethoxybenzoic acid (14), originally described by Burke and coworkers as an efficient pTyr bioisostere (Burke et al. 1998), resulted in compounds that inhibit PTP1B with K_i values of 12 and 2.8 μ M, respectively (Bleasdale et al. 2001). Further development of these series led to compounds 15 (K_i =3.4 μ M), 16 (K_i =0.25 μ M), and 17 (K_i =0.12 μ M). Prodrug analogs of compounds 16 and 17 were recently shown to enhance uptake of 2-deoxyglucose by L6 myotubes (Larsen et al. 2003).

To assist further structure-based design efforts, PTP1B was co-crystallized with compounds 15 and 16 (Bleasdale et al. 2001). The binding mode of compound 15 resembles that reported for the cyclic FOMT-based peptide (10) (Groves et al. 1998) with the exception that the phenyl ring of compound 15 is positioned about 1 Å closer to the P-loop, thereby causing steric hindrance contributing to the open conformation of the WPD loop. However, important hydrogen bonds to the main chain nitrogens of the P-loop are missing compared to the known PTP1B-peptide complexes. Superficially, the pTyr bioisostere in compound 16 is quite similar to OBA (1) (see below). However, superimposition of the crystal structures of compound 16 and OBA in complex with PTP1B reveals significant differences (Fig. 10). While OBA shares most of the interaction points of pTyr in the active site, com-

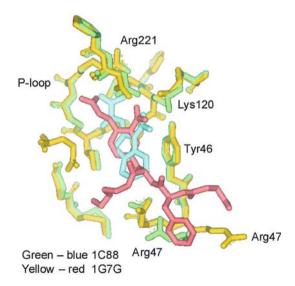


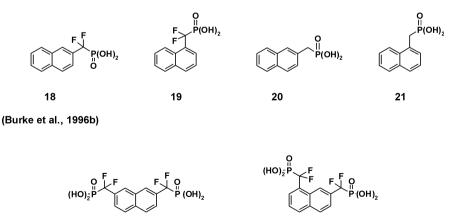
Fig. 10 Superimposition of 1C88 (PTP1B shown in *green*; OBA in *blue*) and 1G7G (PTP1B shown in *yellow*; compound **16** in *red*). OBA shares most of the interaction points of pTyr in the active site, whereas compound **16** only forms contact with Arg221 in the P-loop

pound **16** only forms contact with Arg221 in the P-loop, and the position normally taken up by the phosphate group of pTyr is essentially unoccupied by the inhibitor, but replaced with two water molecules that promote the binding between the ligand and the P-loop (Bleasdale et al. 2001). Similar to the situation with PTP1B complexed with OBA, the WPD loop is closed and the *o*-carboxy group interacts with Lys120 and Asp181. Although not reported by these authors, it is likely that the binding of compound **15** to PTP1B is pH independent, whereas the binding of compound **16**—like OBA (Andersen et al. 2000)—is pH dependent due to its interaction with Asp181.

9.2 Non-peptide Inhibitors

9.2.1 Phosphorus-Containing Inhibitors

Based on molecular modeling of several phenylalanine phosphonate analogs, Burke and colleagues took the first step towards the design of low molecular weight, non-peptide phosphatase inhibitors (Kole et al. 1995b). Fifteen different aryl-containing phosphonates were synthesized and tested for their ability to inhibit PTP1B and serine threonine phosphatases (PP1 and PP-2A). Two difluoro-naphthalene-based phosphonates [1- or 2-substitued (difluoro-naphthalen-1(2)-yl-methyl)-phosphonic acids—compounds 18 and 19] were found to inhibit PTP1B and PP-2A equally well (Fig. 11). In contrast, the non-fluorinated counterparts are poor inhibitors (compounds 20 and 21). Detailed enzyme kinetic analyses or structural information was not reported in this study. However, it was quickly followed by a report of the X-



23

22 Fig. 11 Non-peptidyl phosphorus PTP inhibitor leads

ray structure of PTP1B in complex with compound **18** (difluoro-naphthalen-2-yl-methyl)-phosphonic acid (Burke et al. 1996b; Ye and Burke 1996). Based on this structure, it was hypothesized that the addition of a hydroxyl group at the 4-position of the naphthyl ring could replace a water molecule and thereby allow H-bond formation to Tyr46 and Lys120. Indeed, addition of this hydroxyl group increased the affinity twofold, i.e., structure-based design of non-peptide PTP inhibitors was born. Further development of this concept was leading to compounds with K_i values in the low micromolar range [(Groves et al. 1998) pdb code1BZC].

At Merck-Frosst these studies were extended, and it was found that introduction of two difluoromethylenephosphonic (DFMP) groups into naphthalene-based compounds significantly increased the potency against PTP1B (Wang et al. 1998). For the first time it was demonstrated that selectivity could be obtained by different, simple substitutions. Thus, within this series one compound (22) was five- to sixfold more potent against PTP1B (K_i =16 μ M) than CD45 and another compound (23) showed a fivefold preference for CD45 (K_i =9 μ M) over PTP1B. Further elaborations on this approach led Zhang et al. to develop a highly selective PTP1B inhibitor with similar potency as compound 22, i.e., $K_i \sim 1 \mu$ M (Taing et al. 1999).

9.2.2 Non-phosphorus Inhibitors

Although introduction of the DFMP group represented a significant step forward in regard to potency, it was also realized that such compounds are not cell permeable, probably due to the highly polar nature of the dianionic DFMP group (Kole et al. 1995b). The first attempt to replace phosphonate or the DFMP group in non-peptidyl inhibitors with less polar groups was reported by Burke et al. (1996a) (compounds 24 and 25, Fig. 12). Unfortunately, the inhibitory capacity of these compounds was not reported. However, they inspired another research group to search for phosphate bioisosteres that were more effective than the malonyl and CF-malonyl groups. It was

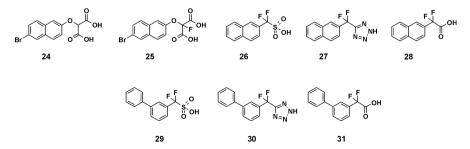


Fig. 12 Non-peptidyl, non-phosphorus PTP inhibitor leads

thus demonstrated by Taylor and coworkers that CF₂-sulfonate (**26** and **29**), CF₂-tetrazole (**27** and **30**), and CF₂-carboxylate (**28** and **31**) were more effective phosphate bioisosteres than CF-malonyl, although not as effective as DFMP (Kotoris et al. 1998) (Fig. 12). In particular, compounds **29** and **31** were investigated in more detail and found to be competitive inhibitors of PTP1B with K_i values of 49 μ M and 98 μ M, respectively.

9.2.3 Simultaneous Optimization Based on Bioinformatics

Our own efforts on inhibitor development started, as mentioned, with a search for a general inhibitor that potentially could be used as a synthetic scaffold or starting point for development of selective inhibitors of all PTPs (see schematic representation of this strategy in Fig. 6). OBA was identified as an active site-directed lead structure showing classical, competitive inhibition of several PTPs (Andersen et al. 2000; Sect. 8). Although negatively

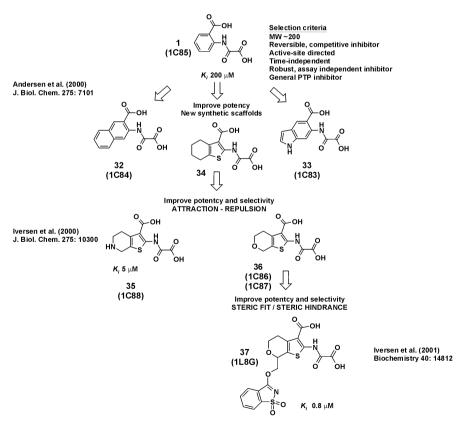


Fig. 13 Bioinformatics-based lead optimization

charged, this compound fulfilled all selection criteria with pH dependency as the only potential limitation. OBA was found to inhibit five out of six PTPs, i.e., it was indeed a general PTP inhibitor. The first step in lead optimization was to improve the general potency of OBA. Inspired by the observations with phosphonates (Sect. 9.2.1), naphthyl- (32) and indole-based (33) OBA (1) analogs were synthesized and found to have significantly improved affinities for several PTPs (Fig. 13). Of note, PTP-LAR, the only PTP that was not recognized by OBA, was inhibited by compound 32, thus demonstrating that the key structural elements of OBA, i.e., the two carboxy groups bound directly, or through a carbonylamino group, to an aromatic ring represents a true, general PTP inhibitor (Andersen et al. 2000). These early studies also showed differences in inhibitor profiles of compounds 32

and 33, thus demonstrating that subtle differences in the active site pocket can be used to obtain some selectivity. Mostly for synthetic reasons, we replaced the second aromatic ring with a saturated ring (compound 34), which showed an almost identical inhibitory profile as that of compound 32 against a number of different PTPs (Andersen et al. 2000; Iversen et al. 2000). A number of other useful synthetic scaffolds derived from OBA were recently reported (Andersen et al. 2002).

9.2.4 Attraction—Repulsion

As described above, bioinformatic analyses revealed that residues 47, 48, 258, and 259 in combination form selectivity-determining regions. Based on modeling of compound 34 in PTP1B, we hypothesized that introduction of a basic nitrogen in this compound at position 6 would place it in an ideal distance from the acidic side chain of Asp48 to allow a salt bridge formation in PTP1B and in other PTPs, whereas with an asparagine in the equivalent position this would cause repulsion. This is an important hallmark in the approach that we have taken: simultaneous improvement of binding to the desired target (in this case by attraction) and decreased binding to non-target enzymes (repulsion). As hypothesized, compound 35 showed an about eightfold increase in potency compared to compound 34 with a K_i of ~5 μ M at pH 7 and with more dramatic selectivity against most other PTPs (Iversen et al. 2000) (Fig. 13).

To validate the attraction/repulsion approach, we made reciprocal mutations in PTP1B (as representative for PTPs with an aspartic acid in position 48) and in PTP α (as representative for Asn48-PTPs). By introducing an asparagine in position 48 in PTP1B, the K_i value for compound 34 dropped about 100-fold. Conversely, the introduction of an aspartic acid in the equivalent position of PTP α resulted in significant binding, although weak compared to wild-type (wt) PTP1B. Thus, these mutants unequivocally demonstrated the importance of Asp48 in PTP1B as a selectivity-determining residue (Iversen et al. 2000). Further, by replacing the basic nitrogen in compound **35** with an oxygen atom yielding compound **36** we obtained roughly threefold increase in potency against $PTP1B_{D48 N}$ compared to wt PTP1B and a similar decrease in potency against $PTP\alpha_{N48D}$. The crystal structures finally confirmed the enzyme kinetic observations with Asp48 and Asn48 in the rotamer 3 position, 2.8 Å (i.e. hydrogen bond distance) from the basic nitrogen (wt PTP1B/compound **35**) and the pyran oxygen (PTP1B_{D48 N}/compound **36**), respectively. In contrast, the wt PTP1B and comound **36** complex showed Asp48 in the rotamer 1 position, i.e. 5.8 Å away from the pyran oxygen due to repulsion. Thus, the combined analyses of these compounds and PTP mutants clearly demonstrated the importance of Asp48 in PTP1B as a selectivity-determining residue and provided further support for the simultaneous optimization strategy (Iversen et al. 2000).

9.2.5 Steric Fit—Steric Hindrance

In PTP1B, Met258 and Gly259 form the bottom of a cleft-like structure positioned between the second aryl phosphate binding site and the active site pocket. We have termed this region the 258/259 gateway, which allows easy access to the active site of PTP1B. In other PTPs with bulky side chains in position 258, the gateway is blocked. Using a number of analogs of the D-A-D-E-pY-L peptide and the reciprocal mutational approach we showed that residue 259 was a key determining residue in PTP1B and PTP α (Peters et al. 2000; pdb code 1GFY). We next decided to use this information in inhibitor design by making compounds that would bind simultaneously to the active site and the 258/259 gateway in PTP1B and TC-PTP, but not to PTPs with bulky residues in position 258. Compound 36 was used as a scaffold in these studies to allow direct evaluation of the influence of substituents on binding to the gateway. Several compounds were synthesized, and compound 37 with a saccharine-based side chain is an example of an inhibitor that efficiently address the 248/259 gateway with a K_i value of 0.6 μ M and 1.1 μ M against PTP1B and TC-PTP, respectively (Iversen et al. 2001) (Fig. 13). Importantly, reciprocal mutational analysis with PTP1B, and with $PTP\alpha$ and PTPH1 as representatives for enzymes with closed gateways, provided evidence for the general applicability of this approach. Indeed, we had simultaneously introduced a very high degree of selectivity against a broad spectrum of PTPs. Significantly, this selectivity was also found against two PTPs that contain an aspartic acid in position 48. Finally, the crystal structure of PTP1B in complex with compound 36 was used to determine the exact binding mode of the saccharine moiety (pdb code 1L8G).

9.3 Addressing Site 2: Specificity Against TC-PTP

Since the original discovery of the second aryl phosphate binding site, site 2 (Puius et al. 1997), and its potential relevance for recognition of substrates with tandem pTyr (Salmeen et al. 2000; Myers et al. 2001), several attempts have been made to design small molecule inhibitors that simultaneously address the active site and site 2 by passing through the 258/259 gateway (Fig. 14). Both peptidyl and non-peptidyl approaches have been utilized. In an extension of their studies on F_2 Pmp analogs, the

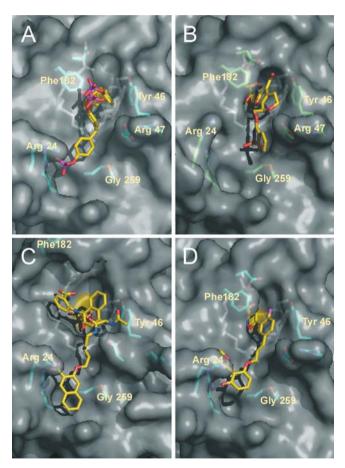


Fig. 14A–D Addressing site 2—X-ray crystallography. Moving from identification of site 2 (A—1AAX) and the 258/259 gateway (B—1L8G) to design of inhibitors that simultaneously address the active site and site 2 by passing through the gateway (C—1NNY and D—1Q1 M). The position of the WPD loop is indicated by the position of Phe182. The figures were drawn with PYMOL (see http://pymol.sourceforge.net/)

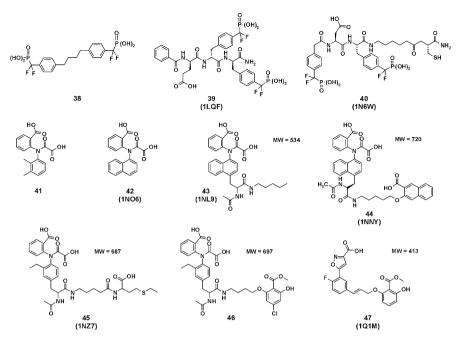


Fig. 15 Addressing site 2 chemical structures

Merck-Frosst group synthesized several compounds with different sizes of linkers connecting two F₂Pmp moieties, as exemplified with compound 38 (Taylor et al. 1998) (Fig. 15). However, although some improvement was obtained in potency, this seemed to be independent of the length of the linker, and it was therefore unlikely that the second aryl phosphate-binding region had been addressed. This was later confirmed by protein X-ray crystallographic studies that, as expected, showed binding of the proximal F₂Pmp moiety to the active site of PTP1B, whereas the distal F_2 Pmp extended into the solvent and interacted with the side chain of Arg47 via a water molecule (Jia et al. 2001). The same group also used peptidyl approaches with two F₂Pmp moieties to address the second aryl phosphate-binding site in PTP1B (Desmarais et al. 1999; Asante-Appiah et al. 2001). Compound 39 is a low nanomolar, selective inhibitor of PTP1B and TC-PTP (Asante-Appiah et al. 2001), which was shown to bind to PTP1B similarly to compound 38 with interaction to Arg47. Although compound 39 does not address the second aryl phosphate binding site, a comparison of this compound in complex with PTP1B and the complex of PTP1B and the tandem phosphorylated pTyr IR peptide reveals important information regarding the structural requirements for compounds that address this site. In the PTP1B-IR peptide complex, the side chain of Arg1164 forms a cation- π interaction with the side chain of Phe182. This interaction seems to be important for

orientation of the peptide substrate towards the second aryl phosphatebinding site (discussed in detail in Asante-Appiah et al. 2002). Thus, future drug design efforts might take advantage of cation- π or other interactions with the WPD loop to orientate side chains correctly through the 258–259 gateway.

The most potent and selective PTP1B inhibitor reported to date with an impressive K_i value of ~1.8 nM (compound 40, Fig. 15) was also synthesized in an attempt to address the second aryl phosphate binding site (Shen et al. 2001). However, it was recently shown by X-ray crystallography that the distal F_2 Pmp moiety of this compound does not bind to the second site but to an area defined by Lys41, Arg47, and Arg48 (Sun et al. 2003). Nonetheless, this compound represents the most potent PTP1B inhibitor and in addition exhibits roughly tenfold selectivity over TC-PTP.

A different, elegant approach was recently reported by the Abbott PTP group (Szczepankiewicz et al. 2003). Similar to our approach, this group used an oxamic acid-based compound as synthetic starting point, compound 41, which inhibits PTP1B-mediated hydrolysis of pNPP with a K_i value of ~300 μ M. The next step was synthesis of compound 42, which inhibits PTP1B with a K_i value of ~40 μ M. X-ray crystallography revealed that compound 42 was binding to PTP1B in the open conformation (pdb code 1NO6). Incorporation of a diamide chain at the 4-naphthyl position resulted in compound 43 (MW 533) that was about 40-fold more potent than compound 42 with a K_i value of ~1 μ M. From a drug design point of view, it was important that the diamide chain was found to interact with Asp48 and that it extends into the 258/259 gateway, thus pointing towards site 2 (pdb code 1NL9). Using NMR screening with [¹³C]-methionine-labeled PTP1B and following the resonance of Met258, several compounds were identified with weak binding affinity for site 2. One of these compounds was coupled to 43, resulting in the potent compound 44 ($K_i \sim 20$ nM—MW 719). Importantly, the naphthoic acid carboxylate group was positioned 2.6 Å from Arg254 and 2.8 Å from Arg24 (pdb code 1NNY). Although compound 44 contains features (molecular weight; three carboxy groups) that make it unlikely as a drug candidate, this is nonetheless an extremely important step forward towards structure-based design of selective PTP inhibitors. Indeed, using the same synthetic scaffold, this research group was able to synthesize potent PTP1B inhibitors (compound $45-K_i$ 76 nM; compound 46— K_i ~40 nM) with about fivefold selectivity over TC-PTP (Liu et al. 2003c; Xin et al. 2003). Using the structural information obtained in these studies, the same research group set out to design selective and cellpermeable PTP1B inhibitors of smaller molecular size and with one carboxylic acid only (Liu et al. 2003b). Again, the inspiration and starting point was the oxamic acid-based inhibitors. A series of heterocycle carboxylic acids were screened with NMR for N-phenyloxamic acid mimetic properties. Using X-ray crystal structure-based assembly, one promising N-phenyloxamic acid mimetic candidate was in turn combined with a fragment that was previously found to address site 2, thus yielding compound 47. Importantly, the molecular weight of compound 47 is only 412 Da, it is cellularly active, and it shows about 30-fold selectivity against TC-PTP. The low molecular weight should allow room for future improvement of the potency of this relatively weak PTP1B inhibitor ($K_i \sim 7 \mu M$), while still retaining the selectivity and cellular activity.

10 Bringing It All Together: Future Challenges

During the last few years there has been significant progress in the development of PTP inhibitors. Using structure-based design approaches, several groups have shown that it is possible to synthesize highly potent and selective non-phosphorus, non-peptide inhibitors of PTP1B. However, at this point these achievements seem to have been reached at the expense of appropriate pharmacokinetic properties, including cellular uptake. Therefore, the next wave within the field of PTP inhibitors is likely to be focused on improvements in this respect.

Furthermore, although very significant progress has been achieved with respect to design of selective PTP1B inhibitors (see Sect. 9), it remains to be demonstrated that drug-like PTP1B inhibitors can be made that do not recognize TC-PTP. TC-PTP is the closest homolog to PTP1B, sharing about 74 percent identity at the amino acid level in the catalytic domains. TC-PTP exists both as a 48-kDa endoplasmic reticulum-targeted form (TC48) and a 45-kDa nuclear form (TC45). Since both PTP1B and TC48 are bound to the endoplasmic reticulum via their C-terminal extensions, it might be speculated that these two PTPs serve similar, perhaps even overlapping, functions. Indeed, in a recent publication it was suggested that TC-PTP is involved in the regulation of insulin signaling (Galic et al. 2003). However, it should be noted that TC-PTP knockout mice, in contrast to PTP1B knockout mice (Elchebly et al. 1999; Klaman et al. 2000), die a few weeks after birth, whereas heterozygous mice seem to have normal lifespan (Ibarra-Sanchez et al. 2000). At present, it is therefore unclear if PTP1B inhibitors that also recognize TC-PTP will be beneficial or harmful. To provide the structural framework for design of inhibitors that are selective for either PTP1B or TC-PTP, we have recently reported the X-ray structure of apo TC-PTP (Iversen et al. 2002). Of note, and as indicated above, two groups have provided structurebased evidence that inhibitors that are selective for PTP1B over TC-PTP are within reach (see Sect. 9.3).

Another challenge facing the PTP field is the fact that PTPs themselves seem to be regulated by covalent modifications, e.g., phosphorylation. In particular, modifications close to the active site may influence not only substrate, but also inhibitor binding. As an example, recent studies indicate that the activity of PTP1B may be influenced by phosphorylation of Ser50 (Ravichandran et al. 2001), which is positioned close to the selectivity-determining region defined by residues 47–48 and 258–259. It is quite likely that the position of the side chains of these residues will be influenced by a bulky, charged phosphate group on Ser50. Since all structure-based design activities up to this point have been carried out with catalytic domains produced in *E. coli*, it is suggested that future design efforts include testing of lead compounds either in appropriately covalently modified recombinant enzymes or in a relevant cellular context. It should also be mentioned that the activity of PTPs may be greatly influenced by domains outside the catalytic domains, and again this has to be taken into account in future PTP inhibitor development programs.

As has become apparent, almost all activities so far have been directed towards development of selective PTP1B inhibitors. However, several other classical PTPs and dual-specificity PTPs may be important novel drug targets, indicating that the focus on general PTP inhibitors may be a powerful platform for the discovery of novel therapeutics. Consistent with this concept, we have been able to use the original scaffold, OBA, as a template for synthesis of highly potent inhibitors of PTP β with significant selectivity over PTP1B, and pharmacological characterization of such compounds may reveal new biological insights and potential clinical applications (Lund et al. 2004).

Finally, it should be, emphasized that the discovery of the transient formation of sulphenyl-amide as described in Sect. 6 may represent an exiting new avenue for structure-based design of PTP inhibitors. The significant conformational changes imposed by the sulphenyl-amide in the active site prevent binding of pTyr substrates and probably all currently known active site-dependent inhibitors. The formation of the sulphenyl-amide is reversible, and it is believed to occur in vivo after stimulation of receptor-tyrosine kinases, resulting in H_2O_2 formation. Therefore, compounds that bind to the sulphenyl-amide form of PTPs, and not to the wild-type enzymes, are expected to inhibit already-activated signaling pathways only, thereby possibly providing an additional level of specificity and limiting the risk of side effects.

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Biological Validation of the CD45 Tyrosine Phosphatase as a Pharmaceutical Target

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Abstract The CD45 phosphotyrosine phosphatase is expressed on all nucleated haematopoietic cells and plays an important role in regulating immune receptor signalling thresholds. CD45 substrates include Src tyrosine kinase family members, and in T cells both p56^{lck} and p59^{fyn} are substrates. Src kinases such as p56^{lck} have negative regulatory C-terminus Tyr phosphorylation sites that are phosphorylated by the c-Src kinase and dephosphorylated by CD45, thereby switching p56^{lck} into a fully functional mode. Active p56^{lck}, and to a lesser extent p59^{fyn}, are involved in coupling the T cell antigen receptor to the intracellular signalling pathways that lead to T cell development and activation. In CD45^{-/-} mice, the threshold for receptor signalling is high and there are significant defects in thymic development resulting in a greater than 90% reduction in the repertoire of mature peripheral T cells. Those CD45^{-/-} T cells that do exit to the periphery are markedly non-responsive to mitogens. The p56^{lck} kinase also has an activating autophosphorylation

site that can be dephosphorylated by CD45 under some circumstances. However, the overall regulatory actions of CD45 on p56^{lck} function in the context of T cell antigen receptor coupling are positive, and this is confirmed by restoration of the $CD45^{-/-}$ phenotype with the *lck*^{Y505F} transgene. Up to eight CD45 isoforms are generated by alternative splicing of which at least five are expressed on lymphocytes at significant levels. Differential CD45 isoform expression is tightly controlled during lymphocyte development and activation, but putative differences in the molecular actions of the different isoforms remain poorly understood. CD45 is an attractive therapeutic target as a means to suppress T cell activation, of relevance in autoimmunity, protection against organ graft rejection and inflammation. CD45 may also be of use in cancer therapies. Current therapeutic strategies utilise CD45 monoclonal antibodies or phosphatase inhibitors. Increasing knowledge of CD45 structure and function will facilitate a more systematic and rational approach to drug design.

Keywords CD45 · Tyrosine phosphatase · Inhibitor · CD45 isoform · Monoclonal antibody

1 Introduction

The CD45 phosphotyrosine phosphatase (previously known as the leucocyte common antigen, LCA) is expressed on all nucleated haematopoietic cells and plays an important role in regulating receptor signalling thresholds in several types of immune cell. The molecule comprises a large heavily glyco-sylated ectodomain (391–552 amino acids) and an extensive cytoplasmic tail (700 amino acids) containing homologous domains 1 and 2 (Fig. 1). Domain 1 contains the protein tyrosine phosphatase (PTPase) activity, whereas the role of domain 2 appears to be largely regulatory. Alternative splicing generates eight or more isoforms that differ markedly in their ectodomain structures and glycosylation profiles.

An extensive range of reviews on CD45 is available (Thomas 1989; Trowbridge and Thomas 1994; Alexander 1997; Ashwell and Doro 1999; Thomas and Brown 1999; Alexander 2000; Justement 2001; Alexander 2003; Hermiston et al. 2003). This chapter will focus on the biological validation of CD45 as a pharmaceutical target and on the various approaches that have been used to inhibit CD45 activity and function.

2 CD45 Regulates Receptor Thresholds

2.1 CD45 and Lymphocyte Development

The first indication that CD45 has a positive role in regulating signals mediated by antigen receptors came from the investigation of T cell lines and T cell clones that lack CD45 expression. In these contexts it was found that

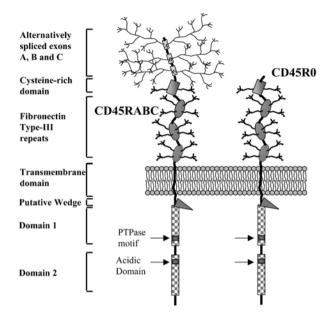


Fig. 1 Domain structure of the CD45 molecule. The extensive CD45 ectodomain is characterised by three fibronectin type III (FN-III) repeats, a cysteine-rich domain and the three alternatively spliced exons A, B and C at the N-terminus. The ectodomain is heavily glycosylated, mainly N-linked in the FN-III and cysteine-rich regions and O-linked in the A, B and C exon-encoded regions. The CD45R0 isoform lacks the A, B and C exon-encoded regions. The protein tyrosine phosphatase (*PTPase*) signature motif VHCSAGVGRTG is located in the C-terminal portion of *domain 1*. Domain 2 contains a characteristic 24 amino acid acidic sub-domain and a partial PTPase signature motif, but is not thought to display PTPase activity

stimulation of the T cell antigen receptor (TCR) no longer induced intracellular activating signals, demonstrating that the signal transduction coupling threshold of the receptor was greatly increased in the absence of CD45 (Pingel and Thomas 1989; Koretzky et al. 1990). Later, these findings were extended and confirmed by the generation of three CD45-targeted mouselines in which CD45 exons 6, 9 or 12 were deleted to generate mice in which nearly all the CD45 was absent (exon 6) (Kishihara et al. 1993) or was completely absent (exons 9 and 12) (Byth et al. 1996; Mee et al. 1999). The low levels of CD45 that remain expressed on the surface of peripheral T cells in the exon 6-targeted mice (Kong et al. 1995b) may in fact have a marked effect in regulating TCR signalling thresholds: low levels of CD45 (as little as 6%–8% of wild-type levels) are sufficient to restore T cell development and functions (Ogilvy et al. 2003). In mice completely lacking CD45 expression there are striking defects in thymic development so that only about 5% of the normal numbers of mature T cells exit to the periphery (Byth et al.

1996). Three distinct defects occur during development: first, there is a partial defect in the transition from CD25⁺CD44⁻ to CD25⁻CD44⁻ cells lacking CD4 and CD8 expression, such that the CD25⁺CD44⁻ subset accumulates in the absence of CD45; second, there is a marked failure of positive selection of CD4⁺CD8⁺ thymocytes, explaining the reduced numbers of mature CD4⁺ and CD8⁺ T cells that exit to the periphery (Byth et al. 1996); third, there is a partial defect in negative selection at the CD4⁺CD8⁺ stage of differentiation, the extent of the defect depending on the avidity of the selecting antigen (Conrov et al. 1996; Wallace et al. 1997; Mee et al. 1999). The reduced transition from CD25⁺CD44⁻ to CD25⁻CD44⁻ cells is readily explained by defects in the signals mediated by the pre-TCR, the immature form of the TCR that is required for this maturation step (Pingel et al. 1999). A further thymic defect that occurs in the absence of CD45 is a marked increase in the basal apoptosis of the CD4⁺CD8⁺ subset: since this population comprises the bulk of the thymus, this survival failure presumably explains much of the 50% reduction in size of the CD45^{-/-} thymus (Byth et al. 1996). Stimulation of CD45^{-/-} thymocytes in vitro using a CD3 monoclonal antibody (mAb) has shown that intracellular signals are typically reduced by 50%-80% (Stone et al. 1997). This contrasts with the virtual ablation of TCR signal transduction observed in most CD45-deficient T cell lines. In fact, optimal TCR stimulation can induce calcium signals in CD45^{-/-} thymocytes to levels comparable with wild-type cells, although the twofold increase in TCR expression on CD45^{-/-} CD4⁺CD8⁺ thymocytes (Stone et al. 1997) may compensate for defects in signalling to some extent. Taken together, these findings suggest that only a very potent CD45 inhibitor, with efficacy higher than 90%, would be likely to have any effect on murine thymic differentiation.

B cell development is less affected in the absence of CD45 than T cell development. Whereas the early stages of B cell differentiation appear to be normal, the maturation from IgM^{hi} IgD^{hi} (immunoglobulin-M^{hi}immunoglobulin-D^{lo}; T2) cells into the IgM^{lo} IgD^{hi} phenotype typical of follicular B cells is impaired (Byth et al. 1996). The thresholds for B cell selection events are altered in CD45^{-/-} mice in a manner analogous to the changes observed in the T lineage. In CD45^{-/-} mice back-crossed to mice carrying immunoglobulin genes specific for hen egg lysozyme (HEL), the circulating HEL autoantigen which mediates negative selection in wild-type mice instead positively selects HEL-binding B cells, leading to their accumulation as long-lived IgD^{hi} cells (Cyster et al. 1996).

The few patients who have been described to date lacking CD45 expression all display a severe combined immunodeficiency (SCID) (Kung et al. 2000; Tchilian et al. 2001) similar to that noted in the $CD45^{-/-}$ mice. Overall, therefore, observations from both mouse and human are consistent with the idea that CD45 plays a dominantly positive role during lymphocyte development by increasing the threshold for antigen receptor signal transduction.

2.2 CD45 and Lymphocyte Function

The B cell antigen receptor (BCR) is almost completely uncoupled from proliferative signals in $CD45^{-/-}$ B cells (Benatar et al. 1996; Byth et al. 1996), correlating with defects in calcium signalling and in activation of the Erk-2/ pp90-Rsk pathways (Benatar et al. 1996; Cyster et al. 1996). The transition from IgM^{hi} IgD^{hi} cells into the IgM^{lo} IgD^{hi} phenotype is known to require a range of BCR-mediated intracellular signals, including Btk and Vav 1/2 regulation (Doody et al. 2001; Meade et al. 2002), so this maturation defect is most likely explained by the high threshold for BCR signalling in the absence of CD45 at this developmental stage.

Nevertheless, CD45-deficient mice appear to mount normal immune responses to T cell-independent antigens (Kong et al. 1995a), whereas T cell-dependent responses are severely defective (Kong et al. 1995a; Ogilvy et al. 2003). Adoptive transfer studies have also shown that B cells from exon 6-targeted mice (Kong et al. 1995a), as well as from exon 9-targeted mice (N. Holmes, unpublished), are capable of isotype switching in response to T-dependent antigens, providing T cells are present from $CD45^{+/+}$ mice. Therefore the defect in antigenic responses in CD45-deficient mice is localised to the T cell compartment.

As a result of the shifts in the thresholds of selection events during T cell differentiation in the CD45^{-/-} thymus, self major histocompatibility complex peptides (MHC-peptides) that would normally have caused deletion of self-reactive TCRs now result instead in positive selection, leading to a high proportion of T cells expressing autoreactive TCRs (Trop et al. 2000). However, the development of autoimmunity in $CD45^{-/-}$ mice is not as aggressive as this finding might suggest, presumably because the few T cells that exit to the periphery are markedly non-responsive to antigenic stimulation. TCR stimulation in CD45^{-/-} purified mature peripheral T cells results in virtually no proliferation (Stone et al. 1997) and the production of cytokines such as interleukin-2, interleukin-4 and interleukin-10 (IL-2, IL-4 and IL-10) is also much reduced (L. Perry and D.R. Alexander, unpublished). Therefore the results so far indicate that CD45-deficient mature T cells are remarkably nonresponsive to stimulation, although the generation of a normal mature T cell repertoire by conditional silencing of the CD45 gene will be important in order to distinguish clearly between the consequences of aberrant development and defects caused directly by the absence of the CD45 molecule.

2.3 CD45 and Macrophages

CD45^{-/-} macrophages display abnormally high integrin receptor-mediated adherence compared with wild-type controls, correlating with the matura-

tion of fewer bone marrow-derived macrophages when differentiated in vitro (Roach et al. 1997). Therefore in contrast to the situation with antigen receptors, CD45 appears to exert a predominantly negative effect on integrin signalling. Consistent with this observation, in a CD45-deficient T cell line, enhanced adhesion to fibronectin via the $\alpha_5\beta_1$ (VLA-5) integrin but not via $\alpha_4\beta_1$ (VLA-4) has been noted (Shenoi et al. 1999).

2.4 CD45 and Mast Cells

Investigation of mast cell function in CD45 exon 6 targeted mice has suggested that CD45 is essential for degranulation triggered via the high-affinity immunoglobulin E receptor (Fc- ϵ R1) (Berger et al. 1994). Similar results have been obtained using CD45-deficient cell lines (Zhang and Siraganian 1999; Murakami et al. 2000). On the other hand, stimulation of the Fc- ϵ R1 receptor in mast cells from either exon 9-targeted (N. Holmes, unpublished results) or exon 12-targeted (V. Tybulewicz, unpublished results) mice has not revealed any obvious defect in primary mast cell degranulation when CD45 is completely absent. Therefore the precise role of CD45 in Fc- ϵ R1 signal transduction remains to be clearly resolved.

2.5 CD45 and Natural Killer Cells

A marked increase in the numbers of natural killer (NK) cells has been noted in the spleen of both CD45 exon 6 and exon 9-targeted mice. Nevertheless, the NK cells appeared to have normal cytotoxic activities and could also differentiate normally into lymphokine-activated killer cells upon culture with IL-2 (Yamada et al. 1996; Martin et al. 2001). In addition, using the exon 9-targeted mice lacking CD45 expression, some subtle defects have been noted in the development and function of NK cells and of NK-T cells (Martin et al. 2001). For example, the cytotoxic NK cells were functionally unable to secrete interferon (IFN)- γ . In addition to NK cells there is a subset of lymphocytes that share receptor structures common to both T and NK cell lineages, the NK-T cells. These can be divided into two distinct subsets, those that are CD4⁺ or CD4⁻CD8⁻ and that develop in a thymus-dependent manner, and a CD8⁺ population that are thymus-independent in their development. In exon 9-targeted CD45^{-/-,} mice, the NK-T cell population was maintained in the intestinal intraepithelial lymphoid compartment but not in the spleen and was found to comprise the thymus-independent CD8⁺ subset. Functionally these cells were unable to secrete IL-4 in response to TCR ligation. Therefore CD45 is required for the development of thymus-dependent NK-T cells, and for normal cytokine secretion in both NK cells and in the CD8⁺ NK-T cell subset.

3 CD45 Substrates

3.1 The Src Tyrosine Kinases

The only molecules that have to date been unambiguously identified as substrates for CD45 are members of the Src family of tyrosine kinases. The Src member that has received most attention is p56^{lck}, a kinase mainly restricted in expression to the T-lineage. In fact, the regulation of p56^{lck} by CD45 is sufficient to explain most if not all the results obtained using CD45-deficient cell lines and knockout mice. The p56^{lck} kinase, as with other members of the Src kinase family, is regulated by a wide range of factors. These include: localisation of the kinase in reference to the plasma membrane; binding of proteins containing Pro-rich motifs to the p56^{lck} SH3 domain; engagement of its SH2 domain by pTvr residue-containing proteins; autophosphorylation of the activatory Tyr-394 residue; and phosphorylation by the C-terminal Src kinase (Csk) of the inhibitory Tyr-505 residue. In the absence of CD45 in membranes in vitro (Mustelin et al. 1989; Mustelin and Altman 1990), in T cell lines (Ostergaard et al. 1989; McFarland et al. 1993; Sieh et al. 1993) or primary T cells (Stone et al. 1997), p56^{lck} becomes hyper-phosphorylated at Tyr-505. This causes the kinase to adopt a 'closed conformation' in which the pTyr-505 residue forms an intramolecular association with its SH2 domain (Sieh et al. 1993; Stone et al. 1997). This version of the kinase lacks optimal activity and, perhaps more importantly, could render the p56^{lck} SH2 and SH3 domains less available to engage exogenous proteins. One candidate protein for such interactions is pTyr-319 in the linker region of ZAP-70 which has been proposed to engage with the p56^{lck} SH2 domain (Pelosi et al. 1999). Upon dephosphorylation of pTyr-505 by CD45, p56^{lck} then adopts an 'open conformation', with a modest increase in kinase activity, and with its SH2 and SH3 domains now fully available for exogenous liaisons. It should be noted that dephosphorylation of pTyr-505 is not the only way in which such an open conformation is promoted. For example, a proline motif-containing peptide in the CD28 cytoplasmic tail can bind to the p56^{lck} SH3 domain (Holdorf et al. 1999) and the Herpes saimiri viral Tip protein can disrupt the relatively weak interaction between the $p56^{lck}$ SH2 domain and pTyr-505 (Hartley et al. 1999), in both cases leading to increased p56^{lck} functionality. The idea that dephosphorylation of p56^{lck} pTyr-505 by CD45 is a physiologically relevant action of this PTPase is supported by the finding that in $CD45^{-/-}$ mice back-crossed to mice expressing a mutant lck^{Y505F} transgene, there is significant restoration of T cell development and reversal of the increased apoptosis that characterises CD45-1/-CD4⁺CD8⁺ thymocytes (Pingel et al. 1999; Seavitt et al. 1999).

Mutational analysis of p56^{lck} has shown that autophosphorylation of the Tyr-394 residue plays a stronger positive role in promoting the enzyme activity of the kinase in comparison with the relatively weak inhibitory effect of Tyr-505 phosphorylation (Doro et al. 1996). In some CD45-deficient transformed cell lines (Doro et al. 1996) as well as murine CD45^{-/-} thymocytes (Baker et al. 2000), increased phosphorylation at Tyr-394 has been observed, suggesting that CD45 also has the capability to dephosphorylate this residue. Indeed, the overall p56^{lck} kinase activity in the CD45^{-/-} thymus is higher than in the wild-type (Doro and Ashwell 1999), consistent with the idea that it is the pTyr-394 site that is most potent in regulating activity. Dramatic confirmation that CD45 could dephosphorylate p56^{lck} pTyr-394 in vivo came from the observation that aggressive T cell lymphomas develop in $CD45^{-/-}p56^{lck-Y505F}$ mice, caused by the conversion of the mutant $p56^{lck-Y505F}$ into an oncogene by increased Tyr-394 phosphorylation in the absence of CD45 (Baker et al. 2000). It therefore appears that in the CD45deficient T cell, pools of p56^{lck} may exist that are either hyper-phosphorylated at Tyr-505, or at Tyr-394, and perhaps at both sites simultaneously.

Recent analysis of the 'immune synapse' that is formed when MHC-peptides engage the TCR has given some clues as to how CD45 might be involved in regulating the two different regulatory phosphorylation sites of p56^{lck}. The engagement of antigen receptors by ligand results in the formation of a highly structured ensemble of molecules at the receptor contact point known as 'the immune synapse', 'immunological synapse' or 'supramolecular activation cluster' (SMAC). All these terms refer to the same entity. It is thought that the immune synapse is a consequence of initial signalling events and provides a mechanism to facilitate a prolonged and carefully orchestrated transmission of intracellular signals during the period of time required for full lymphocyte activation. As Fig. 2 illustrates, selective repertoires of molecules characterise the central and peripheral regions of the immune synapse (cSMAC and pSMAC, respectively) (Monks et al. 1998). The immune synapse is a dynamic system in which molecules rapidly traffic between its various regions.

The important question as to the precise relationship between CD45 localisation and the immune synapse has not yet been fully resolved, partly because the results obtained have varied somewhat depending on the model system utilised. A critical question is how CD45 localises in relation to its $p56^{lck}$ kinase substrate. Initially in T cells $p56^{lck}$ appears to co-cluster in the cSMAC but then moves to the periphery within a few minutes of receptor stimulation (Monks et al. 1998; Ehrlich et al. 2002; Holdorf et al. 2002). At the same time, the immune synapse appears to be replenished with more $p56^{lck}$ from intracellular stores (Ehrlich et al. 2002). The question, then, is how CD45 relates to this moving target. Some imaging studies using either T cells (Leupin et al. 2000) or B cells (Batista et al. 2001) have suggested that CD45 is excluded from the immune synapse. However,

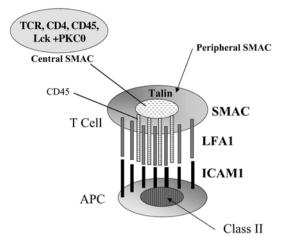


Fig. 2 The location of CD45 in a T cell "supramolecular activation cluster". The T cell antigen receptor (*TCR*) expressed on a T cell engages class II MHC-peptides presented on an antigen-presenting cell (*APC*). At 45 s following engagement the SMAC begins to form, Talin is localised in the central portion of the SMAC (*cSMAC*) and LFA1 is activated and binds to intercellular adhesion molecule (*ICAM*)-1 on the APC. At 3 min the TCR, CD45, CD4, Lck and PKC θ are localised in the cSMAC followed by activation of the p56^{lck} kinase. By 7 min active ZAP-70 tyrosine kinase is found in the cSMAC and increased protein tyrosine phosphorylation in the cSMAC and peripheral SMAC (*pSMAC*), and CD45 has moved out to a region beyond the pSMAC. The model and kinetic data are based on the findings of Freiberg et al. (2002). It is possible that different CD45 isoforms are localised differently and with distinctive kinetics within the various SMAC regions

in a careful kinetic study in which three-dimensional immunofluorescence microscopy was utilised to study the interaction between primary T cells and cognate peptide presented on an antigen presenting cell, both CD45 and p56^{lck} were initially identified in the cSMAC (Freiberg et al. 2002). Then, following 7 min of receptor stimulation, CD45 moved out of the cSMAC to a region peripheral to the pSMAC. Even in the absence of antigen-presenting cells, using a system in which the MHC-peptide ligand was presented to T cells on beads, CD45 was still initially found in the cSMAC in close association with the TCR, showing that recruitment of CD45 to the immune synapse was not caused by an exogenous ligand (Freiberg et al. 2002). In a different study in which planar lipid bilayers containing MHC-peptide and the adhesion molecule intercellular adhesion molecule (ICAM)-1 were used for antigen presentation, CD45 was at first excluded from the cSMAC, but within 10 min was then recruited back to the centre of the contact area (Johnson et al. 2000). The discrepancies between these results may be due to the different experimental systems utilised, in particular the use of lipid bilayers for antigen presentation in contrast to antigen presenting cells. A further intriguing possibility is that different CD45 isoforms (see below) might behave differently with respect to their localisation within the immune synapse, and this question was not addressed in the cited studies.

Overall, although the data are not yet completely clear, a likely scenario is that both CD45 and $p56^{lck}$ are present in the cSMAC at the earliest stage of its formation, enabling CD45 to dephosphorylate $p56^{lck}$ at pTyr-505 and maintain the kinase in a basally functional state (Fig. 2). Following $p56^{lck}$ kinase activation, CD45 then moves to a peripheral region where it may not have continued contact with $p56^{lck}$, so preventing rapid dephosphorylation of pTyr-394 and preserving the kinase in an activated state to facilitate TCR coupling to intracellular signalling pathways (Alexander 2000). In this context, it is of interest that a potent inhibitor of $p56^{lck}$ kinase activity completely inhibited T cell proliferation even when added 1 h after TCR stimulation, suggesting that ongoing $p56^{lck}$ activity is important for the T cell activation process (Ehrlich et al. 2002).

The pharmaceutical relevance of this potentially dual action of CD45 on p56^{lck} is that CD45 inhibitors could, in principle, exert either an activatory or inhibitory effect on the kinase depending on which of the two phosphorylation sites was dominant. Since p56^{lck} Tyr-394 phosphorylation is amplified only upon activation of the kinase, and activation is hindered by hyperphosphorylation at Tyr-505, it seems reasonable to conclude based on the CD45-deficient cell studies that the dominant effect of CD45 inhibition would be a concomitant inhibition of p56^{lck} due to increased Tyr-505 phosphorylation, so preventing the generation of pTyr-394 as a 'second substrate' for CD45. Nevertheless, it cannot yet be excluded that partial inhibition of CD45 might change the ratio between the phosphorylation status of the two sites in subtle ways, and it could eventually emerge that the regulation of this ratio is the key to the development of CD45 as a successful pharmaceutical target.

In addition to p56^{lck}, the p59^{fyn} tyrosine kinase has also been shown to be regulated by CD45 in vivo in both T cell lines (Shiroo et al. 1992; McFarland et al. 1993) and in primary thymocytes (Stone et al. 1997). The adapter protein SKAP-55 may play a role in binding to CD45 and mediating its effects on p59^{fyn} (Wu et al. 2002). The precise function of CD45-regulated p59^{fyn} in coupling the TCR or other receptors to specific signalling pathways has not as yet been completely defined.

It has proved much more difficult to establish unambiguous CD45 substrates in B cells as compared to T cells. Indeed, BCR-stimulated tyrosine phosphorylation events appear normal in $CD45^{-/-}$ B cells. The reason for this may be the greater degree of redundancy in the utilisation of tyrosine kinases by the BCR in comparison with the TCR. Furthermore p59^{lyn}, a further member of the Src family of tyrosine kinases, is involved not only in coupling the BCR to intracellular signals, but also in mediating negative signals via the CD22 co-receptor, so there is scope for compensation between different receptors expressed on the same B cell. In fact, recruitment of the Syk tyrosine kinase to the BCR proceeds normally in a CD45-deficient cellline, even though p59^{lyn} is hyper-phosphorylated at its C-terminus inhibitory Tyr-508 regulatory site and is no longer itself recruited to the receptor (Pao 1997).

Whether p59^{lyn} is the key B cell substrate for CD45 under physiological conditions, explaining the defects observed in BCR signalling, remains controversial. In CD45-deficient B cell lines there is evidence that the kinase is hyper-phosphorylated at both its autophosphorylation and C-terminus inhibitory regulatory Tyr residues, correlating with reduced activity of the kinase (Yanagi et al. 1996). But in a different CD45-deficient B cell line, basal p59^{lyn} kinase activity is normal despite hyper-phosphorylation of p59^{lyn} (Pao and Cambier 1997), whereas in a CD45-deficient sub-clone of the WEHI-231 B cell line, overall p59^{lyn} activity is higher with increased phosphorylation at both regulatory Tyr residues (Katagiri et al. 1999). In contrast to these cell-line studies, dysfunctional regulation of p59^{lyn} in primary CD45^{-/-} B cells has not yet been reported. One possible reason for these conflicting results may be that different pools of Src family kinases are differentially regulated by CD45 and by other tyrosine phosphatases depending on their subcellular localisation (Biffen et al. 1994). Measurements of the 'average' activities and phosphorylation states of kinases by using enzyme immunoprecipitates from whole cell lysates may not sufficiently discriminate between such pools. Therefore in B cells, physiological CD45 substrates have not yet been unambiguously established.

In macrophages from the CD45 exon 6-targeted mice, the p56/p59^{hck} and p59^{lyn} tyrosine kinase members of the Src kinase family are both hyper-active, indicating that these are the likely physiologically relevant substrates for CD45 in this lineage (Roach et al. 1997).

3.2

Other Possible CD45 Substrates

In addition to members of the Src kinase family, several other CD45 substrates have been proposed. For example, CD45 may negatively regulate cytokine receptor coupling to intracellular signals by dephosphorylating the Janus kinases (JAKs) (Irie-Sasaki 2001; Yamada et al. 2002). The JAKs positively regulate cytokine receptor signalling by phosphorylating transcription factors known as the signal transducers and activators of transcription (STATs) which, upon phosphorylation, translocate to the nucleus where they regulate genes involved in the cytokine response. Hyper-phosphorylation of Jak-2 following IL-3 stimulation was noted in bone-marrow-derived mast cells derived from either the exon 6- or exon 9-targeted mice, correlating with increased phosphorylation of STAT-3 and STAT-5 (Irie-Sasaki 2001). Mice lacking CD45 also displayed increased protection against the picornavirus coxsackievirus B3 (CVB3) (Irie-Sasaki 2001), although a similar protection has been noted in $lck^{-/-}$ mice (Liu et al. 2000), so it is possible that increased protection in the absence of CD45 is an indirect consequence of its well-established regulation of p56^{lck}, rather than a reflection of JAK activation.

Other substrates that have been suggested for CD45 include ZAP-70 (Mustelin et al. 1995) and TCR- ζ (Furukawa et al. 1994; Hegedus et al. 1999). It has been suggested that the CD45 D2 domain may be involved in the recruitment of TCR- ζ as a substrate (Kashio et al. 1998). A complication in the assessment of TCR- ζ as a potential CD45 substrate in vivo is that the polypeptide is phosphorylated by CD45-activated p56^{lck}, so in CD45^{-/-} thymocytes, for example, there is a marked reduction in TCR- ζ phosphorylation (Stone et al. 1997). This means that in the absence of CD45, its putative TCR- ζ substrate is not generated normally, so even if it were a substrate it would not be expected to be hyper-phosphorylated. The expression of mutant hyper-active lck^{Y505F} in murine T lymphoma cell lines lacking CD45 expression (Baker et al. 2000) potentially overcomes this problem, but in these cells there is no evidence for TCR- ζ hyper-phosphorylation (D. Higgins and D.R.Alexander, unpublished). A caveat to these findings is that the TCR- ζ polypeptide expression is very low in these cells, and therefore its hyperphosphorylation might be difficult to detect. Overall, therefore, the possibility that CD45 dephosphorylates one or more of the six potential pTyr sites in TCR- ζ cannot yet be excluded.

4 CD45 Isoforms

CD45 is expressed as multiple isoforms resulting from alternative RNA splicing of exons 4, 5 and 6 (also denoted A, B and C, respectively) (Trowbridge and Thomas 1994; Alexander 1997). The exons 4-6 encode only about 50-60 amino acids, each located at the ectodomain N-terminus, but the effects of splicing out these alternative exons is amplified by the loss of their multiple O-linked glycosylation sites (Fig. 1). The cytoplasmic tail is identical in all CD45 isoforms. In theory alternative splicing of exons 4-6 generates up to eight different isoforms, but in practice only five of these isoforms have been reported as proteins expressed at significant levels in lymphocytes: CD45RABC, CD45RAB, CD45RBC, CD45RB and CD45R0, varying in molecular weight from 180 to 240 kDa (Rogers et al. 1992; Fukuhara et al. 2002). Alternative splicing (by mechanisms reviewed in Hermiston et al. 2003) has been most studied in the T lineage, where it is under tight regulatory control during thymic development and in the activation of mature T cells. For example, CD45R0 is up-regulated on CD4⁺CD8⁺ thymocytes, but then declines following positive selection with up-regulation of higher molecular weight isoforms (such as CD45RB in the mouse) in CD4⁺ and CD8⁺ thymocytes (Fukuhara et al. 2002). Human cord blood T cells that have not previously been exposed to antigen express low CD45R0 levels relative to higher molecular weight isoforms containing the A-exon product. With increasing age the proportion of peripheral T cells expressing CD45R0 gradually increases until it typically reaches 40%-60% of the total repertoire in the adult (Hayward et al. 1989). Indeed, when CD45RA⁺ T cells are activated in vitro they up-regulate CD45R0 and down-regulate the CD45RA⁺ isoforms over a period of several days (Akbar et al. 1988). However, this conversion is never complete, and all T cells express more than one CD45 isoform. It is not, therefore, possible to purify primary T cell subsets from wild-type mouse or human and assess the properties of single CD45 isoforms in isolation. The up-regulation of CD45R0 on antigen-experienced T cells, and upon T cells activated in vitro, has led to the idea that CD45R0⁺ T cells comprise, or contain a population, of 'memory/effector' cells. However, this suggestion remains controversial and the only clear conclusion at present is that in the mature T cell repertoire CD45R0 is a marker for previously activated T cells. There is also evidence suggesting that conversion to a CD45R0^{hi} phenotype is a reversible process, so in vivo it seems probable that CD45R0 is not only a marker for previously activated T cells, but also for T cells stimulated relatively recently.

T cells that accumulate at sites of inflammation generally express high levels of CD45R0. For example, many of the T cells found in the inflamed synovium in rheumatoid arthritis (Matthews et al. 1993) and in inflammatory dermatoses (Markey et al. 1990) are in a pseudo-activated state and express high levels of CD45R0, HLA-DR and other activation markers. Interestingly, several therapeutic reagents have now been shown to selectively deplete CD45R0⁺ T cells in patients. For example, alefacept is a fusion protein consisting of the first extracellular domain of lymphocyte function-associated antigen 3 (LFA-3, CD58), fused to the human IgG1 hinge, CH2 and CH3 domains, that binds to CD2 expressed on T cells. The fusion protein prolongs cardiac allograft survival in primates (Kaplon et al. 1996) and has recently been licensed following phase II and phase III clinical trials for the treatment of chronic plaque psoriasis (Ellis and Krueger 2001), a disease characterised by infiltration of memory/effector type CD45R0⁺ lymphocytes. Alefacept has been shown to reduce the number of CD45RO⁺ cells in psoriasis patients, a finding that correlated with improvement of the psoriatic lesions (Ellis and Krueger 2001); and selective killing of CD45R0⁺ rather than CD45RA⁺ T cells by autologous NK cells in the presence of alefacept has also been demonstrated in vitro (Cooper et al. 2003). Therefore the selective depletion of activated/memory type T cells appears to be a promising avenue for anti-inflammatory therapeutic intervention.

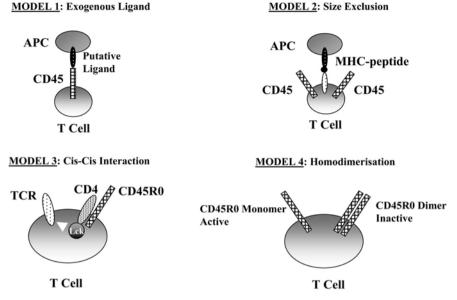


Fig. 3 Four possible models to explain the molecular actions of CD45 isoforms

As Fig. 3 illustrates, four models have been suggested to explain the different molecular actions of CD45 isoforms, and these models will be briefly reviewed. None of the models are mutually exclusive.

4.1 The Exogenous Ligand Model

It is a reasonable assumption that CD45 might have exogenous ligands, either in soluble form or expressed on other cells such as antigen presenting cells, and that such ligands could vary between different CD45 isoforms. However, until now, no physiologically relevant CD45 ligands have been described. Although CD45 binds effectively to lectins, not surprisingly in light of its abundant oligosaccharide branches, such interactions have not as yet been demonstrated to play a role in vivo in modulating the actions of CD45.

4.2 The Size Exclusion Model

An equally reasonable assumption is that the larger CD45 isoform ectodomain might sterically hinder the presentation of MHC-peptides to the TCR, whereas the relatively small CD45R0 ectodomain would exert little or no hindrance (Davis and van der Merwe 1996). This possibility is closely related to the question as to how and when CD45 localises within the 'immune synapse' as discussed in Sect. 3.1. An intriguing possibility is that CD45 isoforms differentially localise within the immune synapse, thereby modulating the speed and/or intensity of T cell responses. For example, in a variant of the size-exclusion model, CD45R0 could be present at the earliest formation of the cSMAC, promoting the activation of p56^{lck}, whereas CD45 isoforms with larger ectodomains might be excluded from the cSMAC, thereby reducing the speed of response in the initiation of TCR signalling. However, the mere size of the CD45 ectodomain per se does not seem to be the main arbiter of CD45 function. Thus, in one study three chimaeric proteins, each containing the CD45 cytoplasmic tail but having small (Thy-1), intermediate (CD2) or large (CD43) ectodomains, were expressed in a CD45-deficient T cell line (Irles et al. 2003). The expectation in this investigation was that the large ectodomain would prevent superantigen-mediated TCR stimulation by preventing the close apposition of the ligand. Unexpectedly, however, the CD43-CD45 chimaera was found to be most efficient at restoring TCR signalling, whereas the chimaeras with small ectodomains were poor in this respect. This correlated with the localisation of the CD43-CD45 chimaera in lipid rafts, whereas chimaeras with smaller ectodomains were excluded from rafts. This study therefore provides no support for the size-exclusion model.

It is important to resolve the issue of CD45, and more particularly of CD45 isoform, sub-cellular localisation in relation to the micro-architecture of the immune synapse, because disruption of the correct location of a specific CD45 isoform might prove to be a potent method for disrupting CD45 function. This possibility will be assessed further below.

4.3

The Cis-Cis Interaction Model

There is an extensive literature describing the association of CD45 with other proteins in *cis-cis* interactions at the cell surface (the earlier literature is reviewed in Alexander 1997). Reconstitution of a CD45-deficient murine cell line with CD45 isoforms suggested that the CD45R0 isoform promoted greater IL-2 secretion upon engagement of the TCR with the cognate MHC-peptide as compared to other isoforms (Novak et al. 1994). Co-capping experiments in these cells revealed preferential CD4-CD45R0 association (Leitenberg et al. 1996). However, more detailed capping and co-immunoprecipitation studies indicated a basal association of CD45R0 with the TCR independent of CD4 expression and suggested that co-capping of CD4 with CD45R0 was mediated by this prior CD45R0-TCR association (Leitenberg et al. 1999). Nevertheless, CD4-CD45 association has also been described in primary CD4⁺ T cells (Mittler et al. 1991; Bonnard et al. 1997). In a further study in which fluorescence resonance energy transfer (FRET) analysis was used to investigate cis-cis interactions, different CD45 isoforms were transfected into a CD45-deficient T cell line expressing the TCR, CD4

and CD8. The results suggested that CD45R0 preferentially associated with CD4 and CD8 relative to the CD45RBC and CD45RABC isoforms (Dornan et al. 2002). The CD45R0–CD4 association correlated with up-regulated protein tyrosine phosphorylation events under both basal and TCR-stimulated conditions. These studies suggest a model in which the juxtaposition of CD45 PTPase activity in relation to its CD4- or CD8-associated p56^{lck} kinase substrate promotes the action of the kinase, thereby up-regulating TCR-stimulated intracellular phosphorylation events.

Attractive as such a model may be, one problem with such cell line studies is that the glycosylation status of CD45 isoforms and their associating glycoproteins may not be the same as in primary cells, resulting in associations that do not accurately reflect those found under more physiological conditions. To address this question, CD45^{-/-} mice have been reconstituted with specific CD45 isoforms. In one study it was shown that either CD45R0 or CD45RABC transgenes, driven by a proximal *lck* promoter, restored T cell numbers to near normal in lymph nodes, while no restoration of T cell populations in the spleen was found (Kozieradzki et al. 1997). In contrast, the Vav promoter has more recently been utilised to generate several lines of transgenic mice expressing either CD45R0 or CD45RB on the CD45^{-/-} background in all haematopoietic lineages (Ogilvy et al. 2003). When expressed at levels comparable with the total CD45 expression found in hemizygous CD45^{+/-} mice, thymic development was restored to apparently normal levels and reconstitution of mature T cells in both the lymph nodes and spleen was observed. In fact expression of CD45R0 at a level only 6%-8% of the total wild-type CD45 expression level was sufficient to restore thymic development to levels equivalent to wild-type. Mice expressing either the CD45R0 or CD45RB isoforms at comparable levels were equally efficient in their ability to mount T-dependent antigenic responses. Interestingly, however, neither isoform at this level of expression was able to restore effective B cell maturation, nor BCR-stimulated proliferation, showing that CD45 isoforms play unique roles which differ between the T and B lineages.

Overall, therefore, there is considerable support, based largely on cell line studies, for the *cis-cis* interaction model, but the model remains to be confirmed as being relevant to the function of CD45 isoforms in the whole animal context.

4.4 The Homodimerisation Model

The possibility that homodimerisation of transmembrane PTPases leads to the inhibition of phosphatase activity has received recent attention. An indication that the actions of CD45 might be regulated in this manner was suggested by studies using a chimaeric receptor with an epidermal growth factor receptor (EGFR) ectodomain and a CD45 cytoplasmic tail. This chimaera successfully restored TCR signal transduction in a CD45-deficient cell line, but TCR signalling was largely abrogated upon subsequently dimerising the artificial receptor by the addition of EGF (Desai et al. 1993). Mutation of a putative wedge domain (Fig. 1) within the CD45 tail portion of the chimaera significantly blunted the ability of EGF to inhibit TCR-mediated signals, suggesting that the wedge domain might be critical in promoting homodimerisation (Majeti et al. 1998). Introduction of a point mutation at the same site in CD45 in a mouse model in situ resulted in a severe lymphoproliferative and lupus-like syndrome with autoantibody production, consistent also with de-inhibition of CD45 PTPase activity (Majeti et al. 2000). However, since the point mutation was introduced into the germ-line, CD45 in all haematopoietic lineages presumably carries the same mutation, so the question of which cell-type is responsible for the observed abnormalities is important.

CD45 has been shown to dimerise in several different studies. For example, CD45 was found to dimerise to a small extent after chemical cross-linking of a T cell line (Takeda et al. 1992) and recombinant fragments of the rat extracellular domain can exist as dimers as well as monomers (Symons et al. 1999). Is it possible that CD45 isoforms might differentially homodimerise, thereby inhibiting specific pools of CD45 PTPase activity? Interestingly, in a study using FRET, it was found that CD45R0 preferentially homodimerises on the surface of a T cell line when compared with CD45RBC or CDRABC isoforms (Dornan et al. 2002). Similar findings were obtained using chemical cross-linking and a cysteine dimer-trapping method in which it was also shown that CD45R0 preferentially homodimerises in a manner hindered by sialylation and *O*-glycosylation (Xu and Weiss 2002).

As with the *cis-cis* interaction model, the homodimerisation model is also an attractive hypothesis for explaining the differential actions of CD45 isoforms. However, significant questions remain. For example, it is important to show by direct biochemical assay that CD45 homodimerisation causes inhibition of its PTPase activity. Furthermore, in the FRET study cited above, both CD45R0 homodimers and CD4/CD8-CD45R0 heterodimers were detected on the surface of the same cell, yet the net effect on TCR signal transduction of CD45R0 expression appeared to be positive (Dornan et al. 2002). In addition, in a series of transgenic lines expressing increasing amounts of CD45R0 at the T cell surface, in which comparable numbers of peripheral T cells were observed in the spleen and lymph nodes, a quantitative effect was noted in which T-dependent antigenic responses correlated positively with the CD45R0 expression level (Ogilvy et al. 2003). This result is difficult to explain if the main role of CD45R0 is to exert a negative effect on T cell responses. Therefore further work will be required to determine whether the homodimerisation model adequately explains the putative differential actions of CD45 isoforms in vivo.

Overall, our understanding of the molecular actions of specific CD45 isoforms remains significantly incomplete. Achieving a thorough understanding of the biological reasons for the existence and tight regulation of CD45 isoform expression in immune cells remains one of the outstanding challenges in the CD45 research field.

5 CD45 as a Therapeutic Target

CD45 is an attractive therapeutic target as a means to suppress T cell activation, of relevance in autoimmunity, protection against organ graft rejection, inflammation and in cancer therapies. Numerous attempts have been made by pharmaceutical companies to target $p56^{lck}$ selectively, and to some extent $p59^{fyn}$, in order to induce immunosuppression, but inhibition of CD45 would presumably inhibit the actions of both kinases simultaneously, thereby achieving the same goal more effectively. The putative dual action of a CD45 inhibitor in down-regulating the functions of both kinases is important because there is considerable evidence for redundancy between the two enzymes (Seddon et al. 2000; Seddon and Zamoyska 2002) Thus a selective $p56^{lck}$ inhibitor might not be as potent in suppressing T cell activation as expected, since $p59^{fyn}$ appears able to substitute for the actions of $p56^{lck}$ to a considerable extent. A CD45 inhibitor would overcome this potential problem.

An important question to be addressed in considering CD45 as a target is the suggested dual role for the phosphatase as both an activator and inhibitor of $p56^{lck}$. As discussed in Sect. 3.1, only when $p56^{lck}$ pTyr-505 becomes dephosphorylated does the kinase become fully functional, thereby leading to increased phosphorylation at Tyr-394. In a sense, therefore, the enzyme has its own internal 'security system' with respect to a CD45 inhibitor, since hyper-phosphorylation at Tyr-505 caused by CD45 inhibition would tend to reduce Tyr-394 phosphorylation. Nevertheless, in CD45^{-/-} thymocytes at least, there is a modest increase in $p56^{lck}$ Tyr-394 phosphorylation (Baker et al. 2000), so the possibility cannot be excluded that at some critical level of CD45 inhibition a specific intracellular pool of $p56^{lck}$ might be activated rather than inhibited. This possibility will require careful assessment during any attempt to target CD45 therapeutically.

The biological studies described above suggest that the dominant effect of a CD45 inhibitor would be on the T lineage, since the phenotypic effects of CD45 deficiency in other lineages are relatively mild, at least in the murine context. Based on the mouse models, T-independent antigenic responses would remain intact even in the presence of the most potent CD45 inhibitor, and it is striking that quite low levels of CD45 expression are sufficient to restore immune responses to T-dependent antigens (Ogilvy et al. 2003). Nevertheless, possible side effects of CD45 therapy on cell types such as macrophages and NK cells will require careful assessment.

Two main approaches are being utilised to target CD45: the use of specific antibodies and direct inhibition of its PTPase activity.

5.1

The Use of CD45 Monoclonal Antibodies

There is a very extensive literature concerning the actions of CD45 mAbs on T cells, and earlier publications have been reviewed elsewhere (Alexander 1997). It should first be noted that there is an important difference in the potential mechanism of action of CD45 mAbs, depending whether or not secondary cross-linking antibody is utilised in the experimental system. During the late 1980s and early 1990s many publications appeared claiming that the dominant action of CD45 was negative with respect to TCR signal transduction coupling (reviewed in Alexander 1997). These results were based on experiments in which CD3 mAb against the relatively rare TCR was cross-linked with the very abundant CD45 molecule. Since in many of the cell systems utilised CD3 ligation was required to induce intracellular signals, extensive cross-linking with abundant CD45 thereby produced a 'dilution effect' whereby CD3 ligation became less effective and, not surprisingly, the intracellular signals were inhibited. However, it was later shown using chemically engineered divalent antibodies consisting of F(ab') fragments against both CD3 and CD45, that when this dilution effect is prevented by fixing the CD3/CD45 ratio, then there is no inhibition (Alexander et al. 1992; Shivnan et al. 1992). In fact, under these conditions the enforced juxtaposition of CD45 with the TCR amplifies T cell activation responses, consistent with the dominantly positive effect of CD45 on TCR signal transduction coupling. Therefore results based on cross-linking the abundant CD45 molecule with other receptors using secondary antibody should be treated with caution, particularly in the absence of relevant controls.

By contrast, there are many instances in which specific CD45 mAbs have striking effects on T cells under conditions in which cross-linking is not an issue. In animal models, one of the main uses of CD45 mAbs has been to induce tolerance to tissue transplants. The most extensive studies have been carried out using a CD45RB mAb called MB23G2 that has been shown to induce long-term engraftment of islets tissue into MHC-disparate diabetic mice (Auersvald et al. 1997; Basadonna et al. 1998), to prevent renal allograft rejection (Lazarovits et al. 1996; Zhong and Lazarovits 1998) and to be useful in the treatment of preclinical models of autoimmunity (Zhong and Lazarovits 1998). Tolerance could be adoptively transferred by transfusion of tolerant mouse CD4⁺ splenic lymphocytes into naïve allografted animals (Gao et al. 1999). The induction of tolerance was associated with a partial depletion of peripheral blood lymphocytes and with a shift in CD45 isoform expression on T cells from higher molecular weight isoforms to a proportionally greater increase in CD45R0 (Basadonna et al. 1998). In further studies to investigate the mechanism of action of MB23G2 in tolerance induction, it was found that mAb treatment of mice was followed by a rapid upregulation on T cells of CTLA-4, a receptor that mediates negative signals, inhibiting T cell activation (Fecteau et al. 2001). Administration of a blocking CTLA-4 mAb at the time of transplantation prevented the anti-CD45RB therapy from prolonging the survival of islet allografts, suggesting that CTLA-4 up-regulation was playing a role in tolerance induction. In addition, treatment with cyclosporin A blocked the MB23G2-induced CTLA-4 expression and promoted acute rejection. How the CD45RB mAb treatment causes up-regulation of CTLA-4 on T cells remains an intriguing question.

Rat models have also been developed to investigate the actions of CD45 mAbs in vivo. The rat anti-rat RT7 mAb has been extensively used. In the rat, two allomorphic forms of the RT7 antigen exist, known as RT7^a and RT7^b (previously known as ART-1 and Ly-1) (Wonigeit 1979a,b). In animals expressing the RT7^a allotype, the RT7^a mAb binds to all CD45⁺ cells. Treatment of rats with the RT7^a mAb causes a massive depletion of peripheral leucocytes as well as bone-marrow precursor cells (Dahlke et al. 2002), and this is associated with tolerance induction to fully MHC-mismatched grafts (Ko et al. 2001). Interestingly, mature B cells, although well coated with the mAb, are protected from depletion. Leucocyte depletion showed an identical pattern in complement-deficient as in normal rats, suggesting that complement lysis is probably not important for the depleting effect (Dahlke et al. 2002). However, RT7^a mAbs (IgG2b) of other isotypes were much less effective at depletion, indicating that Fc-receptor-mediated interactions are important. Besides the actions of such mAbs in tolerance induction, depletion of stem cells has potential in the therapy of haematopoietic malignancies and also in the conditioning of bone-marrow for other indications.

The mechanism of action of the RT7^a mAb in depleting leucocytes remains unknown, but CD45 ligation using certain mAbs has been shown to directly induce apoptosis in either murine or human T and B cells (Klaus et al. 1996; Lesage et al. 1997). The ligation of CD45 is associated with its localisation to the detergent-insoluble cell fraction. Results based on expression of CD45 mutants in the CD45-deficient BW5147 thymoma T cell line have suggested that CD45 PTPase activity and in fact most of the cytoplasmic tail are not required for the apoptosis induced by CD45 ligation (Fortin et al. 2002). The role of CD45 in regulating apoptosis has also been suggested by the finding that in CD45^{-/-} CD4⁺CD8⁺ thymocytes there is a marked increase in basal apoptosis (Byth et al. 1996) that is reversed upon expression of the active lck^{Y505F} transgene (Baker et al. 2000), implicating CD45-regulated p56^{lck} in the induction of survival signals. It is, therefore, possible that perturbation of CD45 using specific mAbs might interfere with the ability of CD45-regulated p56^{lck} to maintain survival signals. However, this does not appear to be the mechanism in the BW5147 experimental system, since the CD45 PTPase activity appears to be dispensable (Fortin et al. 2002).

CD45 mAbs have also been widely used with some success in pre-clinical radiotherapy animal model systems to target CD45⁺ cancer cells with various isotopes (Matthews et al. 1992, 1999; Ruffner et al. 2001; Nemecek and Matthews 2002; Sandmaier et al. 2002; Vallera et al. 2003). Isotopes used in these studies include ¹²⁵I, ¹³¹I, ²¹³Bi and ⁹⁰Y. The potential for CD45 mAbs in therapy for haematological malignancies has also been highlighted by animal studies in which the CD45 mAb inhibited the proliferation of cancer cells (Nemecek and Matthews 2002). For example, a CD45 mAb was effective in inhibiting growth of systemically disseminated human non-Hodgkin's lymphoma B cells in SCID mice (Dekroon et al. 1996).

Increasing understanding of the differential molecular actions of the different CD45 isoforms will influence future strategies used for developing CD45 mAbs as therapeutic reagents. For example, if the heterodimerisation model of CD45 isoform actions developed using cell line model systems (Dornan et al. 2002) proves to be relevant to primary T cells, then potentially mAbs could be developed that blocked, for example, the proposed interaction between CD4 and CD45R0, thereby down-regulating T cell activation.

5.2 The Use of Chemical Inhibitors

Until now much of the published data on CD45 PTPase inhibitors has been reported as an adjunct to results presented on the inhibition of other PTPases (Lee and Burke 2003). The non-availability of CD45 crystal structures illustrating PTPase active site interactions with inhibitors has also hindered rational drug design. Nevertheless, certain classes of compounds have been shown to be potent inhibitors of CD45, and their actions in cell-based systems, at least in the T lineage, are consistent with the dominantly positive role of CD45 in regulating receptor signalling.

A variety of reagents, which may be divided into metal-containing or non-metal-containing, exert inhibitory effects on a broad range of PTPases, including CD45, but their lack of specificity render them of less interest in the therapeutic context (Lee and Burke 2003). For example, suramin is a potent inhibitor of CD45, with complete inhibition within 10 min at low micromolar concentrations, but it also inhibits a wide range of other PTPases (Ghosh and Miller 1993). Small molecules such as peroxynitrite (Takakura et al. 1999) and sulphotyrosyl peptides (Desmarais et al. 1998) are also nonspecific CD45 inhibitors

Various classes of phosphorus-containing analogues have been shown to be effective CD45 inhibitors, such as the phosphonate-containing class of small molecule inhibitors. These include nitroarylhydroxymethylphosphonic acid derivatives which demonstrate low micromolar affinity (IC₅₀ values ranging from 2 to 12 μ M) against CD45 together with some selectivity for CD45 when compared to PTP1B (Beers et al. 1997). The arylmethylphosphonic moieties in such compounds act as surrogates of phenylphosphate functionality. In contrast, a variety of compounds have been identified through natural product screens that do not contain phosphorus. For example, the aporphine alkaloid nornuciferine from *Rollinia ulei* extracts shows an IC₅₀ of 5.3 μ M towards CD45 (Miski et al. 1995). Screening of *Streptomyces* extracts led to the discovery of the hydroquinone dephostatin that inhibits CD45 with an IC₅₀ of 7.7 μ M (Imoto et al. 1993; Watanabe et al. 1995). An unsaturated lactam called pulchellalactam was isolated from the marine fungus *Corollospora pulchella* and displayed selectivity in inhibiting CD45 as compared to PTP1B (Alvi et al. 1998).

Small molecule screens have generated some non-phosphorus-containing compounds that show some promise as selective CD45 inhibitors. The benzimidazole derivative TU-572 inhibits CD45 with an IC_{50} of 0.28 μ M (Hamaguchi et al. 2000) and shows considerable specificity in comparison with a range of other phosphatases. The compound was also shown to suppress IgE-mediated anaphylaxis and murine contact hypersensitivity reactions (Hamaguchi et al. 2001). Derivatives of phenanthrenediones have also been found to be potent and reversible CD45 inhibitors at low micromolar concentrations, inhibiting T cell proliferation in biological assays (Urbanek et al. 2001).

Although the achievements in the field of CD45 inhibition have been somewhat modest relative to other PTPases, it is expected that the availability of crystal structures will accelerate the search for biologically relevant and selective inhibitors. A focused effort in this direction may well prove rewarding.

5.3

Other Possible Approaches to Perturbing CD45

Even without a CD45 crystal structure, it is already clear that the cytoplasmic D2 domain plays an important role in regulating the actions of CD45. Thus, mutation of various conserved residues surrounding the Cys¹¹⁴⁴ in the pseudo-active site of domain 2 caused a 50% reduction in the PTPase activity of domain 1 (Johnson et al. 1992), whereas mutation of Glu¹¹⁸⁰ to Gly in domain 2 abrogated PTPase activity completely (Ng et al. 1995). Evidence using recombinant polypeptides in vitro suggests that domain 2 stabilises domain 1 and increases its PTPase activity: the destabilising Glu¹¹⁸⁰ to Gly point mutation affected this stabilisation (Felberg and Johnson 1998, 2000). Furthermore, the spacer region between the two tandem repeats appears to interact with domain 2 (Hayami-Noumi et al. 2000). In functional experiments in which TCR signalling was examined following reconstitution of a CD45-deficient cell line with mutant forms of CD45, substitution of domain 2 with that from the LAR PTPase abrogated the production of IL-2 (Kashio et al. 1998). This defect could not be explained by ablation of the PTPase activity, suggesting a specific role for domain 2.

Taken together, these findings suggest that the targeting of the D2 domain might be an alternative strategy for perturbing CD45 function. Disruption of the normal interaction(s) expected between the D2 and D1 domains might be dynamically equivalent to the actions of a PTPase inhibitor, or even directly cause PTPase inhibition. Although speculative, this approach is worth considering.

6 Conclusions

Considering that the CD45 gene was cloned and its alternatively spliced forms were already recognised in the mid-1980s, the precise functions of the molecule in vivo have proved remarkably challenging to unravel. Nevertheless, the main outline of how CD45 mediates its effects is now clear. The precise molecular actions of the different CD45 isoforms remain poorly understood, and elucidation of their functions may facilitate novel rationales for therapeutic intervention. Without doubt, CD45 remains an attractive pharmaceutical target in order to achieve immunosuppression, and its targeting might prove to have certain advantages when compared with the alternative strategy of inhibiting the p56^{lck} tyrosine kinase.

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Serine/Threonine Protein Phosphatase Inhibitors with Antitumor Activity

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Abstract Recent studies with fostriecin and derivatives of cantharidin suggest that the development of specific, or highly selective, inhibitors of serine/threonine protein phosphatases, notably PP2A, PP4, and PP5, may prove useful for the medical management of human cancer. This chapter will review the discovery and development of natural compounds that were originally shown to have marked antitumor activity and subsequently found to act as potent inhibitors of certain PPP-family phosphatases. The review will focus on two compounds, cantharidin and fostriecin, addressing discovery, molecular mechanisms of action, affects on cultured cell, clinical use, toxicity, plasma pharmacokinetics, and a brief review of data from a phase I human clinical trial.

 $\textbf{Keywords} \ \ Fostriecin \cdot Cantharidin \cdot Phosphatase \cdot Inhibitor \cdot Tumor \cdot Human \cdot Serine$

. Introduction

In eukaryotic organisms, reversible phosphorylation influences the biological activity of many proteins. Phosphorylation occurs commonly on serine, threonine, and tyrosine residues and represents a fundamental mechanism for controlling intracellular events as diverse as metabolism, contractility, membrane transport, gene transcription, cell motility, and protein synthesis. Protein phosphorylation is a dynamic process. Therefore, at any point in time the phosphorylation status of a given protein reflects the combined actions of both the protein kinases and the protein phosphatases that recognize a particular protein as a substrate. Since reversible phosphorylation has been implicated in the control of many critical aspects of cell growth and differentiation, it seems logical that the development of agents to modify the actions of specific protein kinases or phosphatases should prove useful for the treatment of human cancers. Indeed, as discussed in other chapters of this volume, a considerable effort has been devoted to the development of pharmacological agents which regulate the actions of protein kinases. In contrast, until recently the serine/threonine protein phosphatases have been all but ignored as clinically relevant targets for drug development.

One of the reasons serine/threonine phosphatases have been overlooked as targets for drug development is that they were viewed as simple housekeeping enzymes, functioning to "mop up" after the more important "regulatory" kinases. This concept, however, is simply not true, as there is substantial evidence that protein phosphatases, like their kinase counterparts, are highly regulated and dynamic enzymes. Another complicating factor is that, in the human genome, roughly 383 genes encode kinases that act to catalyze the phosphorylation of serine/threonine residues on proteins (Manning et al. 2002). In contrast, as few as 32 genes encode phosphatases that act on serine/threonine phosphoesters. Thus, the serine/threonine phosphatases are outnumbered more than ten to one by their kinase counterparts. This suggests that a compound which inhibits the catalytic activity of a given phosphatase may have a broad range of biological activity, making it difficult to predict possible side effects. Finally, there are concerns that some inhibitors (i.e., okadaic acid and microcystin-LR) may act as tumorpromoting agents.

In spite of these perceived difficulties, it seems that nature has produced a number of compounds that act as potent inhibitors of serine/threonine phosphatases, and some demonstrate substantial antitumor activity. In this chapter, I will review studies leading to the development of phosphatase inhibitors into drugs that have antitumor activity, focusing on two compounds (cantharidin and fostriecin) that have been tested in humans.

1.1 Target Phosphatases for Drug Development

For "rational drug development," one of the first steps is the identification of suitable cellular targets, and to date most targets are proteins. In the development of antitumor agents, many protein kinases have been designated as developmental drug targets based on the roles they play in the regulation of signaling pathways known to affect cellular proliferation. However, for the serine/threonine phosphatases the process has been quite the opposite. That is, compounds with antitumor activity were identified first via conventional methods (i.e., the screening of natural product extracts). The realization that these antitumor drugs acted on protein phosphatases came much later, when investigations into the molecular mechanisms leading to the suppression of tumor progression revealed that the antitumor drugs acted as potent inhibitors of protein phosphatases.

To understand how phosphatase inhibitors with antitumor activity were discovered and developed, it will be helpful to first briefly review the enzymes upon which they act. Traditionally, serine/threonine phosphatases have been placed into four major groups (PP1, PP2A, PP2B, and PP2C) based on their biochemical properties. These enzymes have also been divided into two major gene families [protein phosphatase P (PPP) and protein phosphatase M (PPM)] based on the similarity of their primary amino acid sequence (Cohen 1997). The PPP gene family consists of PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7. It should also be noted that in humans there are actually three genes encoding four isoforms of PP1, two gene encoding isoforms of PP2A, two genes encoding isoforms of PP7, and three genes encoding at least six isoforms of PP2B, with the additional isoforms produced by alternative splicing of mRNA (Honkanen and Golden 2002).

The catalytic subunits of all PPP-family members are structurally similar (Fig. 1). Nonetheless, they can be further classified by their sensitivity to a number of natural compounds that inhibit catalytic activity [i.e., okadaic acid, calyculin A, fostriecin, microcystin, cantharidin, and tautomycin (for review see Honkanen and Golden 2002; Lewy et al. 2002). PP1–PP6 are sensitive to inhibition and contain a similar "toxin"-binding domain near the C-terminal region of the catalytic subunit. In contrast, PP2B and PP7 have regions within the inhibitor-binding domains that are not conserved, and these "inserts" render them less sensitive, or insensitive, to the above listed inhibitors. The PPM family of enzymes, which is not affected by the abovementioned inhibitors, consists of the Mg^{2+}/Mn^{2+} -dependent phosphatases (biochemically classified as PP2C) and the pyruvate dehydrogenase phosphatases (Cohen 1997).

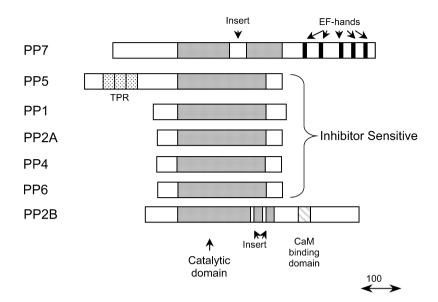


Fig. 1 Homology of serine/threonine protein phosphatases. A schematic representation depicting the amino acid similarity of human PPP-family serine/threonine protein phosphatases. PP1–PP7 contain a common catalytic core domain that is highly conserved. PP1, PP2A, PP4, and PP6 are highly homologous enzymes, differing primarily in their C-and N-terminal regions. PP2B differs in that it contains a Ca^{2+} -calmodulin (*CaM*)-binding domain in its C-terminal region and two small divergent regions (indicated by *arrows* and *open boxes*) in the catalytic domain near the okadaic acid/microcystin binding site. The amino-terminal region of PP5 contains three tetratricopeptide (*TPR*) domains. PP7 differs from all of the other PPase families in that it contains EF-hand motifs in the C-terminal region (indicated by *filled squares*) and a larger (44 amino acid) insert in the catalytic core domain (indicated by an *open box*). The inhibitor-insensitive enzymes contain divergent regions (*inserts*) in the inhibitor binding domain, which have been designated as the $\beta 12-\beta 13$ loop based on the crystal structure of PP1

2 Cantharidin

Although the mode of action was unknown at the time, an inhibitor of PP1– PP6 actually represent one of the oldest treatments for cancer in recorded history. Writings dating back over 2,000 years describe the use of a concoction, "Mylabris", that is produced from the dried body of the Chinese blister beetle (*Mylabris phalerata* or *Mylabris cichorii*) for the treatment of both cancer and warts (Wang et al. 1989). The active constituent of Mylabris is exo, exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride, which is commonly referred to as cantharidin (Fig. 2). Cantharidin is a natural vesicant produced by as many as 1,500 different species of beetles belonging to the order Coleoptera, which produce it as a mechanism for defense.

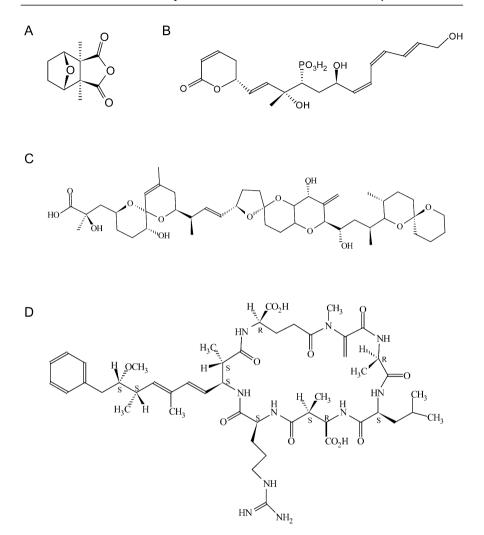


Fig. 2A–D Structures of natural products with inhibitory activity against PP1, PP2A, PP4, PP5, and PP6. A Cantharidin (exo, exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride). **B** Fostriecin {2*H*-pyran-2-one, 5, 6-dihydro-6-[3,6,13-trihydroxy-3-methyl-4-(phosphonooxy)-1,7,9,11-tridecatetraenyl]}. **C** Okadaic acid. **D** Microcystin-LR

2.1 Molecular Mechanism of Action

Although cantharidin was identified as the active constituent of Mylabris in the early 1800s, the first stereospecific synthesis was not reported until the 1950s (Stork 1999), and the understanding of the molecular mechanism underlying its biological actions remained elusive for another 40 years. Then, in a key study it was observed that cantharidin binds with high affinity to a specific and saturable binding site in a cytosolic fraction produced from homogenized mouse liver (Graziano et al. 1997, 1998). Purification revealed that the high affinity cantharidin-binding site was protein phosphatase type 2A (Li and Casida 1992), and further characterization demonstrated that cantharidin acts as a strong inhibitor of PP1 and PP2A and a weaker inhibitor of PP2B (Honkanen 1993). Today we know that cantharidin also affects PP4 and PP5 (Hastie and Cohen 1998; Borthwick et al. 2001; Honkanen and Golden 2002).

Cantharidin is the simplest inhibitor of PP1 and PP2A identified to date, and several studies have addressed the structure-activity relationship of cantharidin by testing the inhibitory effects of derivatives against PP1, PP2A, and PP2B (Sodeoka et al. 1997; McCluskey et al. 2000a,b, 2001; Essers etal 2001). Details of specific modifications have been discussed in recent reviews (McCluskey and Sakoff 2001; Honkanen and Golden 2002; McCluskey et al. 2002b), so here the discussion will be brief. The consensus is that ring opening is needed for inhibitory activity, as attempts to modify the anhydride of cantharidin that prevent ring opening suppress (or eliminate) the inhibitory activity against PP1 and PP2A (McCluskey and Sakoff 2001; McCluskey et al. 2002b). Modification of the 7-O bridgehead is not tolerated, and bulky substitutions at C5, or even modest substitutions at the C1/C4 bridgeheads, suppress inhibitor activity against PP2A (McCluskey et al. 2002b). Unfortunately, to date modifications that substantially improve the potency or the specificity of cantharidin towards PP1/PP2A have not been reported. Nonetheless, modifications of the anhydride moiety on norcantharidins to produce cantharimides have been shown to produce compounds that retain inhibitory activity (McCluskey et al. 2001). This suggests that the synthesis of additional analogs of cantharidin that retain biological activity should be possible, and analogs displaying selectivity (although modest) for PP1 have been reported (McCluskey et al. 2002). The synthesis of fluorinated cantharidin analogs have also been reported (Essers etal 2001). Unfortunately, the effects of fluorination on inhibitory activity were not reported.

2.2 Effects of Cantharidin on Cells

Cantharidin and structurally related compounds have been shown to produce cytotoxic effects in a number of human cell lines, including bone, leukemia, neuroblastoma, ovarian, and colon cancer cells. Cantharidin also has cytotoxic activity against a number of primary tumor cells, with 5–10 μ M cantharidin sufficient to induce death in 24–120 h (Walter 1989; Wang et al. 1990; McCluskey et al. 2000a,b, 2003; McCluskey and Sakoff 2001). In most cells cantharidin can elicit an apoptotic response (McCluskey et al. 2003).

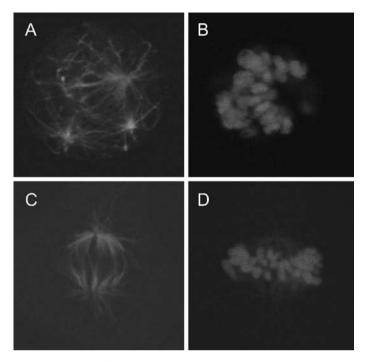


Fig. 3A–D Abnormal spindle formation and chromosome condensation in human tumor cells treated with cantharidin. A549 lung carcinoma cells were treated with 4 μ M cantharidin (A,B) or solvent alone (C,D) were fixed 18 h after treatment, and tubulin was visualized by immunofluorescence following treatment with anti- α -tubulin (primary) and fluorescein isothiocyanate (FITC)-labeled (secondary) antibodies (A, C). DNA was visualized by staining with propidium iodine (B, D)

Still, to date we know little about the molecular mechanisms by which the inhibition of phosphatase activity produces a cytotoxic/antitumor effect.

Studies in CHO cells have revealed that cantharidin treatment induces G2/M-phase growth arrest that is characterized by the formation of aberrant mitotic spindles and occurs prior to induction of apoptosis (Cheng et al. 1998). Similar results have been seen in human lung adenocarcinoma (A549) cells (Fig. 3). G2/M-phase growth arrest has been also noted in glioblastoma cell lines following treatment with norcantharidin, and post-G2/M induction of apoptosis was shown to be dependent on the presence of the p53 gene (Hong et al. 2000). In Drosophila, genetic studies have revealed that the depletion of either PP2A or PP4 results in mitotic failure. The deficiency of PP2A is associated with the uncoupling of nuclear and centrosome cycles and elongated microtubules (Snaith et al. 1996), while depletion of PP4 in embryos results in the absence of microtubules emanating from centrosomes or aberrant microtubules that are unconnected to the centrosomes

(Helps et al. 1998). In *Caenorhabditis elegans*, the suppression of PPH-4.1 (one of two PP4 homologs expressed in *C. elegans*) results in similar aberrant microtubule organization, suggesting that PP4 is needed for the activation of microtubule nucleation at the centrosome (Sumiyoshi et al. 2002). Therefore, in lower eukaryotic organisms the antiproliferative effects of cantharidin may result from the suppression of PP4. Still, until a specific inhibitor for PP2A or PP4 is developed, it is not possible to predict if the mitotic aberrations produced by cantharidin in human cells result for the suppression of PP2A, PP4, or both.

2.3 Clinical Use of Cantharidin

Cantharidin has a long history of use by dermatologists as a treatment for molluscum contagiosum and warts, which continues to this date (Nickolls and Tear 1954; Stoughton and Bagatell 1959; Bagatell et al. 1969; Graziano et al. 1988; Nicholls et al. 1990; Moed et al. 2001). In the United States, the Food and Drug Administration (FDA) approved cantharidin for this purpose prior to 1962. Subsequently, the Food Drug and Cosmetic Act was amended to require drug efficacy data. The data were not supplied by the manufacturers, and cantharidin was removed from the U.S. market (Moed et al. 2001).

As an antitumor agent, cantharidin has been used as a traditional medicine in China for over 2,000 years. It was also used briefly as an antitumor drug in Europe during the 1800s, but by the early 1900s it was generally considered by physicians as too toxic for internal use (Oaks et al. 1960). More recently, the clinical use of cantharidin was found effective against primary hepatoma, but its usefulness was limited by severe toxicity in mucous membranes (Sakoff et al. 1999). Interestingly, in contrast to many types of conventional chemotherapy, cantharidin has been shown to stimulate the production of white blood cells by the bone marrow in a limited number of patients (McCluskey and Sakoff 2001), but renal toxicity has hindered its development for use as an antitumor drug (McCluskey and Sakoff 2001; McCluskey et al. 2002b).

2.4 Toxicity

The clinical testing of cantharidin as an antitumor drug revealed marked kidney toxicity, which has limited further evaluation and suggests that cantharidin is simply too toxic for use in mainstream oncology (McCluskey and Sakoff 2001). Therefore, the amount of human toxicity data from formal clinical trials is limited. However, the erroneous concept that extracts of cantharidin-producing beetles (i.e., "Spanish fly") can be used as an aphrodisiac has resulted in numerous poisonings, and the medical records from patients

suffering from the illicit use of cantharidin provide considerable insight into the toxic effects of cantharidin in humans. If injected, a lethal dose of cantharidin is 10–65 mg, with death usually ensuing in less than 24 h. Ingestion of cantharidin produces painful urogenital tract irritation that can produce priapism in men and pelvic engorgement in women. This likely underlies the myth that cantharidin acts as an aphrodisiac. However, cantharidin poisoning is more commonly characterized by nausea, severe abdominal pain, dysphasia, lumbar pain, urinary tract urgency, shock, convulsions, coma, and death (Wang et al. 1989; Sakoff et al. 1999; McCluskey and Sakoff 2001; McCluskey et al. 2002b).

3 Fostriecin

Fostriecin, also referred to as CI-920, NSC 339638, or PD 110,161, was developed as an antitumor drug by Park Davis, and it entered human phase I clinical trials before it was shown to act as an inhibitor of protein phosphatases. Fostriecin was discovered in the course of a screening program designed to identify new antitumor agents contained in the fermentation broth of an actinomycete. The actinomycete that produces fostriecin was originally isolated from a Brazilian soil sample and subsequently characterized as Streptomyces pulveraceus (subspecies fostreus). Fostriecin, and two structurally related compounds (PD113,270 and PD113,271), were identified as water-soluble components of an extract that was observed to inhibit the growth of murine L1210 lymphoid and P388 lymphocytic leukemia cells in tissue culture without having significant antimicrobial activity (Stampwala et al. 1983; Tunac et al. 1983). Further characterization revealed that all three compounds were phosphate esters containing an unsaturated lactone and a conjugated triene system. Chemical and spectral techniques revealed the structure of fostriecin as depicted in Fig. 2 {2H-pyran-2-one,5,6-dihydro-6-[3,6,13-trihydroxy-3-methyl-4-(phosphonooxy)-1,7,9,11-tridecatetraenyl]} (Stampwala et al. 1983; Hokanson and French 1985). Because fostriecin is unstable above pH 8 and very labile in dilute acid, it can only be purified with isolation steps performed within the pH range of 5.5-7.5 (Tunac et al. 1983; Jackson et al. 1985). Accordingly, fostriecin is purified as a sodium salt. For clinical studies it was formulated with sodium ascorbate to improve stability against oxidation.

3.1 Molecular Mechanism of Action

Initial observations with fostriecin revealed that it inhibited the proliferation of several types of tumor cells both in vitro and in vivo (Stampwala et al. 1983; Leopold et al 1984; Jackson et al. 1985). Therefore, early studies were directed toward understanding how fostriecin's structure influenced its activity. Investigations revealed that fostriecin has cytotoxic activity against a number of tumor cell lines in vitro and marked antileukemic activity in mice (Leopold et al 1984). Tissue culture studies on the structure-activity relationship of fostriecin revealed that the removal of the primary alcohol moiety (PD113,270) had little effect on cytotoxicity against L1210 and HCT-8 cells in vitro. Hydroxylation of the lactone ring (PD 113,271) caused a moderate reduction in activity (Jackson et al. 1985), and removal of the phosphate ester (PD114,631) or opening of the lactone ring (PD 113,027) resulted in a considerable loss of cytotoxic activity (Jackson et al. 1985). Together, these studies suggest that both the phosphate ester and the lactone ring contribute to the biological activity of fostriecin.

Initial biochemical studies revealed that fostriecin produced a marked inhibition of macromolecular synthesis. Treatment of L1210 murine leukemia cells with 10 μ M fostriecin resulted in a decrease in the rate of DNA, RNA, and protein synthesis within 45 min, with maximal inhibition occurring within 60 min (Fry et al. 1984a). Measurement of ribonucleotide and deoxyribonucleotide pools in fostriecin-treated cells indicated that the ATP and 2'-deoxyribo-ATP (dATP) pools did not differ significantly from the control values, and the levels of the other ribonucleoside and deoxyribonucleoside trisphosphates were either elevated or unchanged relative to control cells (Fry et al. 1984a; Jackson et al. 1985). These observations suggested the inhibition of nucleic acid synthesis was not due to precursor depletion. However, no effects on DNA polymerase, RNA polymerase, or enzymes within the purine or pyrimidine biosynthetic pathways were noted, and the mechanism of nucleic acid synthesis inhibition remained elusive (Fry et al. 1984a; Jackson et al. 1985; Scheithauer et al. 1986).

3.2

Inhibition of Cell Cycle Progression

To gain further insight into the mechanism by which fostriecin inhibits tumor cell proliferation, studies designed to determine the phase of the cell cycle at which fostriecin induced growth arrest were conducted (Jackson et al. 1985; Baguley et al. 1989). Treatment of L1210 cells with a cytostatic concentration of fostriecin (1.5 μ M) for 24 h followed by flow cytometric analysis of propidium iodide-stained DNA revealed that fostriecin produced a marked increase in accumulation of cells in the G2/M phase of the cell cycle (Boritzki et al. 1988). In HL-60 and MOLT-4 cells (human promyelocytic and lymphocytic leukemic cells, respectively) 1 μ M fostriecin also resulted in the suppression of progression through the G2-phase of the cell cycle. Treatment with 15 μ M fostriecin, which is cytotoxic, also produced S-phase growth arrest (Hotz et al. 1992a,b). Similar results were observed in CHO cells, where fostriecin induced G2/M-phase growth arrest and, like cantharidin, caused the appearance of multiple aberrant mitotic spindles (Cheng et al. 1998). Effects of fostriecin at higher concentrations (25–500 μ M) also affected cell cycle progression in HL-60 cells, and at these higher concentrations fostriecin triggered endonucleolytic DNA degradation resulting in apoptotic cell death (Hotz et al. 1992a; Graziano et al. 1997). The ability of fostriecin to induce apoptotic cell death at high concentrations (>20 μ M) is addressed in more detail in a recent review of preclinical data obtained with fostriecin (de Jong et al. 1997).

3.3

Inhibition of Topoisomerase II Activity

Topoisomerase II is a nuclear enzyme that binds covalently with DNA, cleaves both DNA strands, passes another part of double-stranded DNA through the break, and then reseals the DNA. Because of its critical role in DNA replication, topoisomerase II is the target for many chemotherapeutic drugs, and the inhibition of topoisomerase II activity arrests growth with a cell cycle distribution similar to that observed following treatment with fostriecin. Early reports indicated that fostriecin inhibited type II DNA topoisomerase (Boritzki et al. 1988). At a concentration of 100 µM, fostriecin completely inhibited the relaxation of supercoiled DNA typically affected by type II topoisomerase (IC₅₀ 40 µM). In contrast to other type II topoisomerase inhibitors, however, fostriecin did not induce DNA strand breaks, suggesting that it did not stabilize a cleavable complex (Boritzki et al. 1988). Rather, it was felt that fostriecin probably inhibited the catalytic activity of sensitive topoisomerases directly. Correlative studies in three small cell lung carcinoma cell lines showing topoisomerase II-related drug resistance compared the concentration of fostriecin that inhibits tumor cell growth with the relative amount of topoisomerase II in the cancerous cells. There was a lack of cross-resistance to fostriecin and a relationship between topoisomerase II activity and fostriecin sensitivity (de Jong etal. 1991). Nonetheless, despite these reports, fostriecin was not found to have an inhibitory effect on topoisomerase II when an in vitro excision-repair system was employed to study the effects of topoisomerase poisoning (Forsina and Rossi 1992). Other investigators conducting studies in CME or K562 leukemia cell lines with topoisomerase inhibitors that do not stabilize DNA-topoisomerase II complexes also failed to observe an inhibitory effect of fostriecin on topoisomerase II (Chen and Beck 1993; Fattman et al. 1996).

3.4 Inhibition of Protein Phosphatase Activity

More recent studies suggest an alternative mechanism of action of fostriecin. In a variety of rodent, simian, and human cell lines, fostriecin inhibits a mitotic entry checkpoint and induces chromosome condensation in interphase cells in a manner comparable to okadaic acid, a known inhibitor of several PPP-family serine/threonine protein (PP1, PP2A, PP4, PP5, and PP6) (Roberge et al. 1994; Cohen 1997; Honkanen and Golden 2002). Further investigations revealed that fostriecin inhibits the activity of both PP1 and PP2A (Roberge et al. 1994; Walsh et al. 1997; Cheng et al. 1998), suggesting that the antitumor activity of fostriecin could also originate from its ability to interfere with the reversible phosphorylation of proteins that are critical for progression through the cell cycle (Roberge et al. 1994; Guo et al. 1995; Ho and Roberge 1996; Walsh et al. 1997; Cheng et al. 1998). Fostriecininduced chromosome condensation correlates with an increase in histone H2A and H3 phosphorylation in mouse mammary tumor cells, and fostriecin-induced hyperphosphorylation of vimentin correlates with the reorganization of intermediate filaments in hamster kidney cells (Ho and Roberge 1996). However, as observed with the effects on topoisomerase II activity, the amount of fostriecin that was associated with enhanced phosphorylation was higher than that associated with antitumor activity: \geq 25 μ M fostriecin was needed to demonstrate histone hyperphosphorylation and \geq 50 µM for vimentin hyperphosphorylation (Ho and Roberge 1996). The higher concentrations may reflect the short time course of the studies (generally less than 4 h), and the intracellular concentration of fostriecin was not determined.

Detailed studies conducted with the purified catalytic subunits of mammalian serine/threonine phosphatases as well as the endogenous enzymes contained in diluted whole cell extracts have confirmed the inhibitory activity of fostriecin against certain eukaryotic serine/threonine phosphatases. When the purified catalytic subunits of PP1 and PP2A from rabbit muscle or bovine brain were used in these inhibition studies, fostriecin displayed potent inhibitory activity against PP2A (IC₅₀=1.5-3.2 nM) and weak inhibitory activity against PP1 (IC₅₀=131 µM) (Walsh et al. 1997; Cheng et al. 1998; Hastie and Cohen 1998). Fostriecin also potently inhibits the activity of PP4 (IC₅₀=3.0 nM) (Hastie and Cohen 1998) and weakly affects the activity of PP5 (IC₅₀ ≥70 µM) (Honkanen and Golden 2002). Fostriecin also inhibits endogenous, divalent cation-insensitive phosphatase activity of diluted whole cell extracts of RINm5F insulinoma (Walsh et al. 1997) and CHO (Cheng et al. 1998) cells, rat and pig myocyte homogenates (Armstrong et al. 1997; Weinbrenner et al. 1997), and homogenates of rabbit ventricular biopsies (Weinbrenner et al. 1997). The divalent, cation-independent phosphatase activity contained in these homogenates is derived primarily from

PP1, PP2A, PP4, PP5, and possibly PP6 (Weinbrenner et al. 1997), and the inhibition kinetics obtained were similar to predicted values for a homogenate composed of these enzymes (Walsh et al. 1997; Weinbrenner et al. 1997; Cheng et al. 1998). The kinetics of fostriecin-induced inhibition of PP2A and PP4 correlate well with the ability of fostriecin to inhibit tumor cell proliferation both in vivo and in vitro, suggesting that additional studies into the relationship of these phosphatases to cell cycle progression is warranted. In addition, the effects of fostriecin related to G2 growth arrest [e.g., the formation of aberrant mitotic spindles (Cheng et al. 1998) and the induction of apoptosis (Gorczyca et al. 1993a,b,c; Hotz et al. 1992a,b)] can be mimicked by other phosphatase inhibitors (i.e., okadaic acid and cantharidin) when applied at concentrations that inhibit activity to a comparable extent (Boe et al. 1991; Hotz et al. 1992a; Ishida et al. 1992; Kiguchi et al 1994; Walsh et al. 1997; McDermott et al. 1998; Cheng et al. 1998).

The relationship between fostriecin actions on topoisomerase II, serine/ threonine phosphatase inhibition, and ultimate cytoxicity are not well understood. Both phosphatase and topoisomerase inhibitors induce G2-growth arrest and apoptosis (Jackson et al. 1985; Boe et al. 1991; Kiguchi et al 1994; Cheng et al. 1998; Gorczyca et al. 1993a,b,c). In addition, in many studies the concentrations of fostriecin needed to induce enhanced phosphorylation of nuclear proteins, inhibition of topoisomerase II, or induction of apoptosis were substantially higher (>20 µM) than those associated with antitumor activity. Cellular uptake may be limited by the transport capacity of the reduced folate transporter, which appears to be required for the cellular uptake of fostriecin (see below). Alternatively, only some of the fostriecin-sensitive phosphatases or topoisomerases may be associated with antitumor activity. At concentrations associated in vitro as well as in vivo with antitumor activity, fostriecin inhibits only half of the divalent cation-independent phosphatase activity contained in RINm5F insulinoma cells and CHO cells (Walsh et al. 1997; Cheng et al. 1998). The inhibition data obtained with the dilute cell extracts is similar to that predicted for a mixture of PP1, PP2A, and PP4; however, because of the lack of truly type-specific inhibitors, the contribution of individual phosphatases cannot be assessed experimentally at this time. Furthermore, although it is clear that fostriecin can induce an apoptotic response, it is still not clear if the response arises from the inhibition of one of the affected enzymes or if it is triggered as a consequence of prolonged G2/M-phase growth arrest.

3.5 Cellular Uptake of Fostriecin

To date, only a few studies have addressed the mechanism of fostriecin uptake. Fostriecin is water soluble, and the evidence available suggests that it enters the cell by a carrier-mediated mechanism (Fry et al. 1984 Jackson et al. 1985; Spinella et al. 1995). Reduced folates (leucovorin and 5-methyltetrahydrofolate) completely protect L1210 cells from the inhibitory growth effects of fostriecin, while folic acid, which is transported by a process distinct from that for reduced folates, does not provide protection (Fry et al. 1984). Therefore, fostriecin appears to enter L1210 leukemia cells via the reduced folate carrier system. Fostriecin also acts as a potent inhibitor of methotrexate influx with a K_i of 30 µM (slope) and K_i (intercept) of 58.8 µM (Fry et al. 1984). Cells that have been made resistant (~70-fold) to the cytotoxic effects of fostriecin by continuous exposure to increasing concentrations of the drug are also cross-resistant to methotrexate and collaterally sensitive to trimetrexate (a hydrophobic antifolate). This fostriecin-resistant cell line also has a severely impaired reduced folate carrier system, exhibiting a methotrexate influx rate of less than 1% of controls.

3.6 Effects of Fostriecin on Cells

Fostriecin has demonstrated marked antitumor activity against a wide spectrum of tumor cells in vitro and in vivo. In cultured tumor cells, fostriecin proved cytotoxic against a number of cell lines (i.e., L1210, HCT-8, P338), and structure–activity relationship studies comparing fostriecin analogs and cytotoxicity indicate that both the lactone ring and phosphate group are important for cytotoxicity of fostriecin in vitro (Lewy et al. 2002). Recently, the total synthesis of fostriecin has been reported (Boger et al. 2001), so analogs for further evaluation should be forthcoming (Lewy et al. 2002).

In mice, fostriecin exhibits potent cytotoxic activity P388 and L1210 leukemias when administered via i.p. injection. Fostriecin was also active against B16 melanomas. In vitro, fostriecin proved efficacious in L1210bearing mice with an IC₅₀ of 0.46 μ M for continuous exposure and 4.4 μ M for a 1-h exposure. Scheduling studies indicated that once-daily treatment for 5- or 9-day periods was more effective then two or three single doses every 4 days, and i.p. dosing at 6.25 mg/kg per day on days 1–9 proved to be curative in the L1210 mouse model (de Jong et al. 1997).

In a clonogenic human solid tumor screening assay, in which fostriecin (2.2 μ M for 1 h) was tested on a number of tumor specimens derived from patients, fostriecin was found to be highly active and compared favorably to 17 clinically used anticancer drugs including etoposide and doxorubicin (de Jong et al. 1997). Fostriecin produced a 50% or greater decrease in tumor colony forming units in 42% of breast, 33% of the ovarian, and 38% of the non-small cell lung cancer samples (de Jong et al. 1997). In solid tumor mice models, fostriecin produced rapid necrosis in advanced implanted murine colon tumors (Baguley et al. 1989). In other studies, fostriecin proved inactive against a range of solid tumors; however, because the tumors were also

insensitive to methotrexate this failure was attributed to the absence of the reduced-folate carrier (de Jong et al. 1997).

Another interesting development is that fostriecin was shown to provide protection to ischemic cardiac tissue comparable to that produced by ischemic preconditioning. Ischemic preconditioning is a phenomenon whereby the heart is protected from an ischemic insult by a previous brief episode of ischemia and reperfusion. Although the mechanism of the protection provided by ischemic preconditioning has remained elusive, several lines of evidence suggest that the activation of protein kinases, such as protein kinase C (Weinbrenner et al. 1998) and the p38 MAPK cascade (Ytrehus et al. 1994) may contribute to this protective response (for review see; Downey and Cohen 1997). To test the potential of fostriecin as an inhibitor of ischemic damage, studies were conducted in perfused, isolated rabbit hearts (Weinbrenner et al. 1997) and isolated ventricular cardiomyocytes from rabbit (Armstrong et al. 1997; Weinbrenner et al. 1997; Armstrong et al. 1998) and pig (Armstrong et al. 1997, 1998). In both pig and rabbit models 0.1-10 µM fostriecin mimicked the protection of preconditioning in both rabbit and pig cardiomyocytes (Armstrong et al. 1997, 1998; Weinbrenner et al. 1997). In isolated, perfused rabbit hearts fostriecin was administered starting either 15 min prior to or 10 min after the onset of a 30-min period of regional ischemia and continuing until the onset of reperfusion. In hearts pretreated with fostriecin, only 8% of the ischemic zone infarcted, which was significantly less than that in the untreated control hearts (33%). Fostriecin also provided significant protection in hearts treated only after the onset of ischemia (18% infarction; p < 0.05 vs control). Together, these studies suggest that type-selective phosphatase inhibitors may also offer promise as infarct size-limiting drugs.

3.7

Human Clinical Trials and Plasma Pharmacokinetics

Because of its spectrum of activity and unique mechanism of action, the Clinical Trials Evaluation Program of the United States National Cancer Institute initiated phase I clinical trials. To determine the pharmacokinetics of fostriecin, a high-pressure liquid chromatographic method to measure fostriecin in plasma samples was developed (Pillon et al. 1994). Fostriecin is extracted from plasma samples with acetonitrile and then dichloromethane. It is then analyzed by HPLC with a mobile phase of 7.5% acetonitrile in 0.067 M phosphate buffer after separation on a reverse phase C_{18} column. The extraction efficiency is reported as 70% and the sensitivity limit 100 ng/ml.

3.7.1 Stability of Fostriecin

In whole blood, fostriecin was found to be stable for the first 2 h, and aqueous fostriecin remained stable for approximately 12 days. Fostriecin concentration in frozen (-20° C) plasma samples decreased after approximately 14 days. The decrease in fostriecin concentration was not affected by adding ascorbic acid to protect the samples from oxidation. At -70° C samples remained stable for approximately 49 days; stability decreased rapidly thereafter (Pillon et al. 1994).

3.7.2 Pharmacokinetics in Rabbits

The pharmacokinetic properties of fostriecin in rabbits were assessed after bolus intravenous injection of 12 mg/m² (Pillon et al. 1994). Disappearance from plasma was best described by a monoexponential equation representing a one-compartment model. The mean distribution space was determined as 4.4 l/m² (~8 times the plasma volume) and the plasma clearance was 302 ml/min/m² (75% of hepatic plasma flow). The plasma half-life of fostriecin was 12.0+/-8.6 min.

3.7.3 Pharmacokinetics in Humans

Only limited data on the pharmacokinetics of fostriecin in humans have been published. The available data are from a phase I oncology study that enrolled 20 patients whose tumors were unresponsive to established treatments (de Jong et al. 1997, 1999). Fostriecin was supplied by NCI as a lyophilized powder and diluted with 0.9% NaCl. It was administered daily as an intravenous infusion for 1 h on each of 5 days at 4-week intervals. The fostriecin doses used were 2, 4, 6.6, 10, 12.2, and 20 mg/m² per day). Three patients were entered into the study at each dose level, with patients receiving the next higher dose when no limiting toxicity was noted at the previous dose. Blood samples were obtained on day 1 prior to infusion, at 30 and 45 min during and at the end of the infusion, and at 10, 20, and 30 min and 1, 1.5, 2, 4, 7 and 17 h after the end of infusion. Urine was collected before the start of infusion and again at 0–2, 2–5, and 15–18 h after the start of the infusion.

A close linear association was observed between drug dose, maximum plasma concentration, and AUC (area under the curve), suggesting linear pharmacokinetics within the dose range investigated. Plasma pharmacokinetics was best described by a two-compartment model. Mean plasma half-life was 0.36 h ($t_{1/2\alpha}$) and 1.51 h ($t_{1/2\beta}$). Mean apparent total body clearance was 2.90 l/h/m² (=48.3 ml/min/m²; 95% CI, 2.24–3.57 l/h/m²). Mean residence time was 1.19 h (95% CI, 0.41–1.97 h). The mean volume of distribu-

tion was 5.64 l/m^2 (95% CI, 2.16–9.11 l/m^2). Only approximately 15% of the fostriecin was excreted in the urine. After approximately 25 min a metabolite, postulated to be dephosphorylated fostriecin, appeared in the plasma and urine of patients receiving more than 12.2 mg/m² fostriecin (de Jong et al. 1998, 1999).

3.8 Toxicity Studies

Preclinical toxicological evaluation of fostriecin was conducted primarily in mice and rats, and to date only a limited amount of toxicity data has been released from human clinical trials.

3.8.1 Toxicity in Rodents

Single-dose intravenous administration of fostriecin at dose levels of 8.8-48 mg/kg resulted in death at a dose of 35 mg/kg (60%) and 48 mg/kg (90%), with most deaths occurring on day 1 or 2 of the study (Susick et al. 1990). The 10% lethal dose (LD_{10}) in mice was 2 mg/m² per day. In most cases, animals exhibited labored breathing, tremors, hypoactivity, hypothermia, and loss of the righting reflex prior to death. Clinical signs were not restricted to animals that died. The majority of animals surviving the study exhibited labored breathing and hypoactivity immediately after dosing, which lasted for approximately 1 week. Major toxic effects were observed primarily at doses exceeding or equal to 17.5 mg/kg and consisted of bone marrow hypocellularity, leukopenia, neutropenia, thrombocytopenia, and diffuse necrosis of lymphoid tissues. The effects were reversible. One target organ was the kidney. Renal effects were observed primarily at doses of 20 mg/kg. Increases in serum BUN, creatinine, and 24-h glucose excretion were noted, but were reversible. After dosing at 10 and 20 mg/kg, 24-h Na⁺ and K⁺ excretion and urine osmolarity decreased. Renal lesions were most prevalent at the dose of 20 mg/kg, and consisted of vacuolization and necrosis of the proximal and distal tubular epithelium at the corticomedullary junction with extension to the medulla.

The effect of schedule and route of administration was also assessed. In mice, fostriecin was both inactive and nontoxic when given orally. Subcutaneous treatment was without antileukemic activity and may have even enhanced toxicity (Jackson et al. 1985). In rats, repeated daily intravenous administration of fostriecin for 5 days at dose levels of 2.5–26.5 mg/kg resulted in death at greater than or equal to 10 mg/kg with similar hematological, bone marrow, lymphoid, and renal changes observed following a single treatment (Susick et al. 1990). All rabbits receiving fostriecin at a dose of 12 mg/m² exhibited a 15-fold increase in serum alanine aminotransferase

(ALT) and a 60-fold increase in serum aspartate aminotransferase (AST). Maximal rises were observed within 4 h after treatment. There was an 11-fold increase in serum bilirubin 24 h after administration, but levels returned to normal within 48 h.

3.8.2 Toxicity in Humans

In the phase I trial, liver and renal toxicities were observed. In contrast to preclinical trials in rodents, almost no hematological toxicity was evident (Susick et al. 1990; de Jong et al. 1998, 1999). Abnormalities of liver function were not associated with symptoms, and often lessened despite continued administration of fostriecin.

To assess renal toxicity, serum concentrations of creatinine, urea, and electrolytes were measured immediately before the start of treatment, daily during the treatment, and twice weekly during treatment-free intervals. In most patients, significant elevations of serum creatinine were observed at the starting dose, and in all patients at doses exceeding or equal to 4 mg/m² per day. But at daily doses above 4 mg/m^2 , a plateau in serum creatinine was reached, with no further increases observed as the dose rose. Serum creatinine levels generally rose after the first dose, reached their maximal level on day 3, stabilized despite continuing drug administration, and recovered in 1-2 weeks (median recovery 10-11 days, range 5-19 days) after discontinuation of the drug (de Jong et al. 1998, 1999). Renal hemodynamics was also measured with clearance studies employing ¹²⁵I-iothalamate and ¹³¹I-hippuran in 8 patients taking doses exceeding or equal to 4 mg/m² per day. Measurements were made at baseline and on day 3 or 4 during and 3 weeks after the first course. The median change in effective renal plasma flow was -23%, and glomerular filtration rate fell by 36%. The filtration fraction decreased in all patients during the first course of treatment. Urinary β 2-microglobulin concentrations increased dramatically (median 150-fold), which is compatible with impaired tubular reabsorption. It was concluded that fostriecin induces a considerable decrease in renal function compatible with renal tubular damage (de Jong et al. 1998, 1999). The rapid recovery and reversibility of renal hemodynamic changes suggest a predominantly functional disorder rather than extensive necrosis. There was no indication of cumulative toxicity (de Jong et al. 1998, 1999); however, the maximal tolerated dose was not determined.

4 Conclusions

Cantharidin appears to be too toxic for use as an antitumor drug in most clinical settings. Still, the in vitro antitumor activity of cantharidin is impressive, and the observation that it stimulates the production of white blood cells by bone marrow seems worthy of further investigation. In addition, the recent syntheses of derivatives that retain inhibitory activity suggest that more selective inhibitors can be developed.

Based on in vitro investigations with fostriecin and the limited data available from the phase I clinical trial, it is not yet clear if blood concentrations of fostriecin predicted to have antitumor activity and achievable at doses of 30-40 mg/m² can be reached in humans. Unfortunately, concerns regarding drug purity and storage stability of the naturally produced material lead to the discontinuation of the trial before dose-limiting toxicities or therapeutic plasma levels were reached. Therefore, further dose escalation is still necessary to define the maximum tolerated dose (MTD) of fostriecin. Nonetheless, the toxicities encountered over the dose ranges investigated to date, and in particular their limited progression with increasing doses, indicate that further dose escalation will be possible (de Jong et al. 1999). The possibility of developing fostriecin for the protection of cardiac tissue under ischemic conditions is also promising. Plasma concentrations (1.5-7.5 µM) of fostriecin achieved with doses of 6.6-20 mg/m² are above the 1 μ M concentration that provided protection to isolated rabbit hearts, and the short halflife and reversibility of the observed toxic effects suggest that even higher doses may be employed clinically for a single treatment. If so, then fostriecin may be useful in limiting the damage to ischemic cardiac tissue. Clearly, additional studies with this interesting molecule will be necessary to provide additional insight into the molecular mechanisms underlying the actions for fostriecin.

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Part IV Inhibitors in Clinical Use or Advanced Clinical Trials

Clinical Immunosuppression using the Calcineurin-Inhibitors Ciclosporin and Tacrolimus

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Abstract T cells play a key role in orchestrating the immune response to an allograft. The discovery of a potent and immunologically specific inhibitor of T cell activation, ciclosporin, dramatically improved the results of renal transplantation and transformed other types of organ transplantation from experimental to standard therapy. The discovery of a second drug, tacrolimus, that was structurally unrelated to ciclosporin but which had an identical mechanism of action, facilitated research which clarified the mechanisms underlying T cell activation. Although these drugs are powerful immunosuppressants, their clinical use is limited by their nephrotoxicity. In transplantation, this has led to their use in lower doses in combination with other immunosuppressive drugs. However long-term nephrotoxicity remains a significant problem, and this has curtailed the use of calcineurin-inhibitors for indications outside the field of transplantation. Ciclosporin and tacrolimus are metabolised by cytochrome P450 3A and are substrates for the P-glycoprotein transporter system. This results in complex pharmacokinetics with large variations in bioavailability and metabolism between individuals as well as a great number of clinically significant drug interactions. Therapeutic drug monitoring has been used to address these issues. For the foreseeable future, these powerful immunosuppressive agents are likely to continue to play a role in organ transplantation. However, newer immunosuppressants that are not nephrotoxic may begin to replace calcineurin-inhibitors for long-term maintenance therapy after transplantation.

Keywords Ciclosporin · Cyclosporine · Tacrolimus · Calcineurin-inhibitor · Immunosuppression · Transplantation · Nephrotoxicity · Autoimmune disease

1 Introduction

Pharmacological immunosuppression is essential for all types of allogeneic organ transplantation (Banner and Lyster 2003). Immunosuppressive therapy is also used to treat a range of autoimmune diseases and will be essential for xenotransplantation if this becomes a clinical reality in the future (Azimzadeh et al. 2003). The efficacy of such immunosuppression depends on the mechanism of action of agents used and their capacity to specifically target those aspects of the immune response which play a key role in the alloimmune response. The discovery of cyclosporin A (hereafter ciclosporin) was a major advance in immunosuppressive therapy because of its specific effect on lymphocytes and their role in the cognate immune response while leaving the functions of the innate immune system and other aspects of the adaptive immune response intact (Borel et al. 1976). The introduction of ciclosporin into clinical transplantation was associated with improved results in the fields of kidney (The Canadian Multicentre Transplant Study Group 1986), heart (Over et al. 1983) and liver transplantation (Starz et al. 1981); in addition, lung transplantation became a successful procedure for the first time (Reitz et al. 1982; Toronto Lung Transplant Group 1986). Ciclosporin was also effective in the control of graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (Deeg et al. 1985) and was found to

Approximate date	Agent	
1960	Azathioprine	
	Corticosteroids	
	Antithymocyte globulin	
1970	Ciclosporin	
1980	Muromonab-CD3	
	Tacrolimus	
1990	Mycophenolate	
	Basiliximab/daclizumab	
	Sirolimus	
2000	Everolimus	

 Table 1
 Introduction of immunosuppressive agents into clinical use for organ transplantation

have a role in the management of autoimmune disease (Ellis et al. 1986; Forre et al. 1987; Tejani et al. 1987). Subsequently, further immunosuppressive agents have been introduced into clinical transplantation (Table 1). One of these was tacrolimus (Kino et al. 1987); it was found to have a similar mechanism of action as ciclosporin but a completely different molecular structure (Sawada et al. 1987). The availability of ciclosporin and tacrolimus facilitated research to establish their mechanism of action (Schreiber and Crabtree 1992). Here, we review the clinical pharmacology of both drugs with particular emphasis on their role in organ transplantation.

2 The Alloimmune Response and Allograft Rejection

Allograft rejection is conventionally divided into three syndromes according to the time of its occurrence; each of these has a different pathogenesis. Acute rejection may involve cellular or humoral mechanisms, although the former usually predominate. Pharmacological immunosuppression based on the calcineurin inhibitors has been effective in the control of acute cellular rejection, which is one of the most common problems early after organ transplantation, but it has had less impact on the other forms of rejection.

Hyperacute rejection was first described in renal transplantation (Williams et al. 1968; Patel and Terasaki 1969; Terasaki et al. 1971) and it has subsequently been recognised to occur in other types of transplant (Smith et al. 1993; Choi et al. 1999; Scornik et al. 1999). Preformed antibodies against antigens of either the ABO blood group system or human leukocyte antigens (HLA) bind to the endothelium of the allograft causing endothelial activation. This causes complement binding and intravascular coagulation which leads to the rapid destruction of the graft (Wu et al. 2003). This rapid

form of rejection can be prevented by ABO matching between the recipient and donor and by screening transplant candidates for anti-HLA antibodies; those who have such antibodies must also undergo a lymphocytotoxic cross match against the donor before a transplant can be safely performed (Smith et al. 1993). When hyperacute rejection does occur, it requires specific treatment aimed at both the removal of the preformed antibodies and preventing the synthesis of further antibody (Bittner et al. 2001; Pierson et al. 2002).

Acute rejection typically first occurs within the first 3 months of transplantation but may occur later. It is predominantly a cellular process (Hall et al. 1978; Rosenberg et al. 1987) and is initiated by helper T cells which recognise either foreign HLA on the surface of cells within the graft (direct recognition) or antigens from the allograft which have been processed by antigenpresenting cells (APCs) of the host (indirect recognition) (Hornick and Lechler 1997). Direct recognition appears to be the most important mechanism in acute rejection. Thus, the T cell plays a central role in orchestrating the immune response to the allograft (Fig. 1) (Rose and Hutchinson 2003).

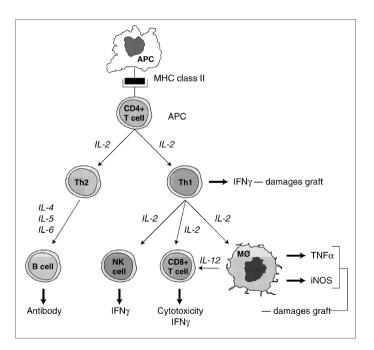


Fig. 1 Role of the helper T cell in orchestration the alloimmune response. Activation of $CD4^+$ T cells results in Th1 or Th2 cells, production of their characteristic cytokine profiles and maturation of effector mechanisms. *APC*, antigen-presenting cell; *MHC*, major histocompatibility complex; *IFN*, interferon; *TNF*, tumour necrosis factor; *iNOS* inducible nitric oxide synthase; *MØ*, macrophage; *IL*, interleukin. (Reproduced by permission from Rose et al. 2003)

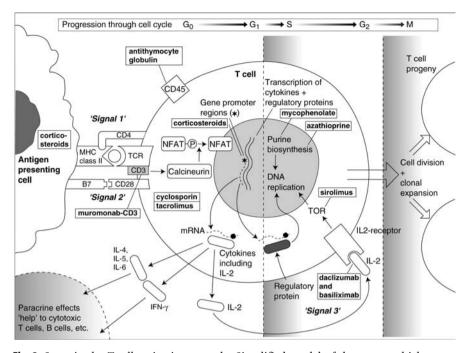


Fig. 2 Steps in the T cell activation cascade. Simplified model of the events which occur during T cell activation. The early, calcium-dependent, phase of activation begins when the T cell receptor (TCR) binds to a complementary MHC class II molecule with an associated peptide in its antigen-presentation grove 'Signal 1'. Full activation also requires a second signal ('Signal 2') which is caused by binding between complementary adhesion molecules on the surface of the antigen-presenting cell and the T cell. Signal transduction from the TCR occurs via the CD3 complex. Subsequent intracellular signalling involves the inositol triphosphate/diacylglycerol pathway and mobilisation of intracellular calcium. This leads to activation of the protein phosphatase calcineurin. Calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) allowing its active moiety to translocate to the nucleus and so bind to the promoter regions of various genes encoding cytokines such as interleukin-2 (IL-2), regulatory proteins and the IL-2 receptor. The pattern of cytokine expression depends on the nature of the T cell (Th1 or Th2) and can lead to either recruitment of cytotoxic T cells and other effector cells or to the provision of help to B cells for antibody production. The expression of IL-2 leads to autocrine stimulation of the T cell. Binding of IL-2 to its receptor initiates a second sequence of intracellular signals involving the mammalian target of rapamycin (TOR) which leads to DNA synthesis and replication and which culminates in cell division. The sites of action of various immunosuppressive agents are shown. Polyclonal antithymocyte globulin is shown as binding to the common leukocyte antigen (CD45) although, in reality, it contains antibodies which bind to many different T cell antigens. Abbreviations: IL, interleukin; MHC, major histocompatibility complex; NFAT, nuclear factor of activated T cells; TCR, T cell receptor; TOR, target of rapamycin; IFN, interferon. (Reproduced by permission from Banner and Lyster 2003)

Binding of the receptor of an alloreactive helper T cell to allogeneic HLA will, if coupled to a secondary signal caused by binding between adhesion molecules on the surface of the T cell and the APC, lead to activation of the T cell via a cascade of intracellular signals (Fig. 2) (Sayegh and Turka 1998). Once activated, alloreactive helper T cells act as a cytokine producing 'engine' which leads to amplification of the immune response through both cell division and the recruitment of other cells (Banner and Lyster 2003). T cells produce a series of cytokines including interleukin (IL)-2, IL-3, IL-4, tumour necrosis factor- α , granulocyte-macrophage colony-stimulating factor and interferon- γ (Wiederrecht, Lam et al. 1993). The effector mechanisms which lead to the destruction of the graft include: the action of cytotoxic T cells which induce target cell necrosis in an MHC-restricted manner via molecules such as granzyme and perforin as well as by inducing apoptosis through the binding of Fas to Fas-ligand (Russell and Ley 2002); less-specific (i.e. non-MHC-restricted) cell killing will also occur by the recruitment of activated macrophages, natural killer cells and eosinophils (Adams and Hamilton 1984; Doody et al. 1994).

Acute humoral rejection is an antibody-mediated phenomenon that is less frequent than cellular rejection. It is caused by the production of antibody directed against antigens in the graft either de novo or as an anamnestic response leading to the resynthesis of a previously formed antibody. Antibody binding to the graft leads to complement-mediated injury with prominent vascular features (acute 'vascular' rejection) (Cherry et al. 1992; Bohmig et al. 2001; Feucht 2003). In severe rejection, cellular and humoral mechanisms often co-exist (Abe, Sawada et al. 2003). Once established, this type of rejection cannot be controlled by conventional immunosuppression based on the calcineurin inhibitors and requires additional specific treatment to remove the antibody and inhibit its resynthesis (Grandtnerova et al. 1995; Hickstein et al. 1998; Garrett et al. 2002).

Chronic rejection is a multifactorial process which is driven by both immune and non-immune mechanisms. Episodes of acute cellular rejection and the formation of alloreactive antibody as well as autoimmune antibodies against non-polymorphic antigens all appear to play a role (Rose and Hutchinson 2003). Immunosuppression based on calcineurin inhibitors has an indirect influence on these processes by reducing the incidence of acute rejection and reducing T cell help to B cells for de novo antibody synthesis. However, it has not overcome the problem of chronic rejection, which remains one of the commonest long-term complications of organ transplantation.

3 Discovery of Ciclosporin and Tacrolimus

Prior to the discovery of ciclosporin, there were a limited number of agents available for clinical immunosuppression (Table 1). Acute rejection was a frequent problem that could lead to graft loss and the use of drugs such as corticosteroids in high doses for a prolonged period, which led to a high incidence of complications, particularly infection. Ciclosporin proved to be an important advance because of its selective and reversible effect on T cell activation (Kahan 1989).

Ciclosporin was discovered in the early 1970s by a team led by Borel working at Sandoz laboratories. The compound was identified from cultures containing the fungi *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Kay 1989). It was found to have immunosuppressive properties in murine models (Borel et al. 1976). Short-term experiments in small laboratory animals did not identify nephrotoxicity as a problem, and so it was a surprise that early clinical experience showed ciclosporin to be nephrotoxic in man (Calne et al. 1978). Subsequent experience and clinical trials confirmed that ciclosporin was an effective immunosuppressive agent and that its use was associated with improved graft survival (European multicentre trial group 1983); however, the issue of nephrotoxicity has remained as one of the main limitations of ciclosporin have been investigated but none has been found to be free of this problem; immunosuppressive effect and renal toxicity appear to change in parallel in this group of drugs (Siga et al. 1991).

Tacrolimus (formerly FK506) was also discovered through a process of screening fungal metabolites. It was isolated from cultures of the soil fungus *Streptomyces tsukubaensis* by researchers at the Fujisawa Pharmaceuticals in 1984. Subsequently, it was found to have similar immunosuppressive properties to ciclosporin but a much greater potency and a completely different molecular structure (Fig. 3) (Kino et al. 1987).

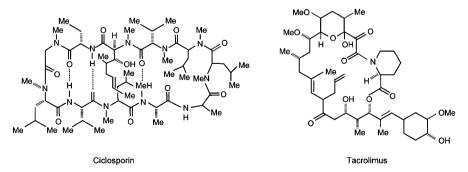


Fig. 3 Molecular structures of ciclosporin and tacrolimus. Despite their common immunosuppressive action, the two drugs have no structural similarity

Structure and Binding to the Immunophilins

Ciclosporin is a hydrophobic cyclic undecapeptide (Fig. 3). In contrast, tacrolimus is a macrolide which has structural similarities to sirolimus (rapamycin) and everolimus but no features in common with ciclosporin. The structural dissimilarity of tacrolimus to ciclosporin and its structural connection with another immunosuppressive agent that has a different mechanism of action, sirolimus, was important in the search for the molecular targets of these agents.

Both ciclosporin and tacrolimus bind to intracellular proteins now known as immunophilins. These are ubiquitous and abundant small intracellular proteins which have been highly conserved during phylogeny (Wiederrecht, Lam et al. 1993). The major receptor for tacrolimus (FK506) is a 12-kDa protein designated FK binding protein 12 (FKBP12). The major receptor for ciclosporin is a 17-kDa protein designated cyclophilin-A (Handschumacher, Harding et al. 1984; Siekierka, Hung et al. 1989).

Despite their different structure, the immunophilins share an enzymatic activity catalysing the isomerisation of peptidyl-proline bonds; they are believed to play a role in protein folding (rotamase activity). Ciclosporin and tacrolimus are inhibitors of their cognate immunophilin's enzymatic activity, but they do not cross-inhibit the other family of immunophilins. The initial hypothesis that inhibition of the immunophilins isomerase activity played a key role in the action of these drugs was refuted by the discovery of congeners which inhibited that activity but that either differed in their mechanism of immunosuppression (sirolimus) or that lacked immunosuppressive activity altogether (Bierer et al. 1990; Dumont et al. 1990). Immunophilins play a role in the clinical pharmacokinetics of these drugs.

5 Mechanism of Action

Most immunosuppressants used in organ transplantation act primarily against the activation programs present within helper T cells (Fig. 2). Ciclosporin and tacrolimus act during the early, calcium-dependent, phase of T cell activation. At therapeutic concentrations, neither drug is cytotoxic and, in vitro, their effect can be reversed by the addition of IL-2). In vitro, to be effective in inhibiting T cell activation, ciclosporin or tacrolimus must be present at the time of T cell receptor ligation or soon afterwards; if the drug is administered more than 6 h after the activation stimulus there is little effect on T cell proliferation (Kay et al. 1983; Kumagai et al. 1988; Tocci et al. 1989).

4

Activation is initiated following the cognate interaction of an alloreactive T cell receptor with a MHC-peptide complex on an APC, coupled with a second signal involving the binding between costimulatory molecules such as B7–1 and 2 on the APC and CD28 on the T cell (Crabtree 1989; Sayegh and Turka 1998). This activates tyrosine kinases, which phosphorylate tyrosine residues of CD3 within the receptor complex (Fig. 4) (Shaw and Dustin 1997). Subsequent intracellular pathways are activated including the inositol triphosphate (IP₃) and diacylglycerol (DAG) pathway. IP₃ stimulates the release of calcium from the endoplasmic reticulum and opens a calcium channel in the membrane, producing an influx of calcium into the cell (Parekh et al. 1997; Shaw and Dustin 1997; Fierro et al. 2000).

Research using ciclosporin, tacrolimus and their analogues clarified the sequence of events that transduce this calcium signal into gene transcription within the T cell (Schreiber and Crabtree 1992; Liu 1993). Affinity matrices of FKBP and cyclophilin were used to identify ligands for complexes of FKBP-tacrolimus and cyclophilin-ciclosporin. Both were found to bind the same set of proteins in a calcium-dependent fashion (Liu et al. 1991). The target proteins were identified as the phosphatase calcineurin and the regulatory protein calmodulin (Klee et al. 1988; Liu 1993). Binding of each drugimmunophilin complex to calcineurin-calmodulin inhibited the phosphatase activity of calcineurin (Liu 1993). In vitro, calcineurin activity was completely abolished by doses of ciclosporin or tacrolimus (hereafter calcineurin inhibitors or CNIs) that inhibited transcription of IL-2 (Fruman, Klee et al. 1992). Calcineurin consists of a catalytic domain coupled to a control region, a calmodulin-binding region and an autoinhibitory domain. Phosphatase activity is activated by the binding of calcium and calmodulin (Kincaid et al. 1986; Hubbard and Klee 1989).

Calcineurin dephosphorylates a number of substrates that play a role in intracellular regulation. The steps linking calcineurin phosphatase activity to the regulation of IL-2 are now understood, but the mechanisms that link this calcium-dependent pathway to effects that do not involve transcription and to the many adverse effects of CNIs are far less clear (Schreiber and Crabtree 1992). However, the correlation of immunosuppressive potency and nephrotoxicity within CNIs and their analogues supports the view that inhibition of calcineurin underlies the toxicity of these compounds (Sigal et al. 1991; Wiederrecht et al. 1993).

Calcineurin dephosphorylates the nuclear factor for activated T cells (NFAT) family of transcription factors. NFAT plays a key role in gene transcription within the activated lymphocyte (Shaw et al. 1988; Luo et al. 1996). NFAT1 is present in the cytoplasm of resting T cells and translocates into the nucleus in response to the calcium signal that follows T cell activation (Figs. 2 and 4) (Shaw et al. 1995; Loh et al. 1996). The event that initiates the nuclear import of NFAT1 is its dephosphorylation by calcineurin (Shaw et al. 1995; Luo et al. 1995; Luo et al. 1995; Luo et al. 1996). By inhibiting the translocation of NFAT to the nu-

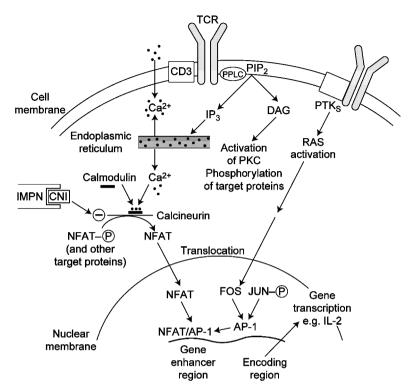


Fig. 4 Mechanism of action of the calcineurin inhibitors. Binding of an MHC-peptide complex to its cognate T cell receptor (TCR) initiates a cascade of signalling events which constitute the early, calcium-dependent, phase of T cell activation. TCR-associated protein tyrosine kinases (PTKs) become activated and lead to the phosphorylation of phosphatidylinositol phospholipase C (PPLC). PPLC then catalyses the breakdown of the membrane phospholipid phosphatidylinositol bisphosphate (PIP₂) to form inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ induces the release of calcium (Ca²⁺) from the endoplasmic reticulum (ER). The phosphatase calcineurin is then activated by the binding of calcium and the regulatory protein calmodulin. Calcineurin dephosphorylates regulatory proteins including the cytoplasmic form of the nuclear factor of activated T cells (NFAT). Dephosphorylated NFAT can then be imported into the nucleus where it binds to the enhancer region of genes such as that for IL-2. DAG, in combination with calcium, activates protein kinase C (PKC), which phosphorylates other regulatory proteins. Other PTKs, activated by cognate binding of the TCR, phosphorylate substrates that activate guanosine triphosphate-binding proteins such as Ras. The Ras pathway links to a mitogen-activated protein (MAP) cascade (not shown) that ultimately leads to the transcription of Fos and the activation of Jun by phosphorylation. Fos and Jun-P combine to form the transcription factor activator protein (AP)-1 which binds to the enhancer region of the IL-2 gene. A number of factors can bind to the enhancer region of the IL-2 gene, including NFAT, AP-1, nuclear factor (NF)- κ B and Oct-1; once bound, they serve to co-operatively activate the transcription of the gene. NFAT plays a key role in gene activation and the calcineurin inhibitors (CNIs) interfere with T cell activation by preventing its translocation into the cell nucleus. Ciclosporin and tacrolimus act as prodrugs which bind to their corresponding immunophilins (IMPN) to form a CNI-IMPN

cleus, calcineurin inhibition prevents the co-operation of NFAT with other transcription factors in enabling the expression of IL-2 and other cytokines (Durand et al. 1988; Wiederrecht et al. 1991; Schreiber and Crabtree 1992).

Experimentally, alternative pathways of T cell activation can bypass calcium-dependent activation of calcineurin, but their relevance to clinical allograft rejection and clinical immunosuppression remains uncertain (June et al. 1987; Lin et al. 1991). The clinical practice of combining a CNI with other types of immunosuppressant may mitigate the effect of such pathways (Banner and Lyster 2003).

6 Clinical Use of the Calcineurin Inhibitors

6.1 Transplantation

6.1.1 Maintenance Immunosuppression

The introduction of the original Sandimmun (or Sandimmune) formulation of ciclosporin as a clinical immunosuppressant for renal transplantation was associated with a marked improvement in patient and graft survival (Canadian Multicenter Transplant Study Group 1983; European Multicentre Trial Group 1983). In the Canadian trial, ciclosporin and prednisone were compared to azathioprine and prednisone; in the European study, ciclosporin monotherapy was compared to azathioprine plus steroids. However, in the Canadian trial, serum creatinine levels were higher in the ciclosporin group, suggesting nephrotoxicity; and in the European study, 24 of 84 patients in the ciclosporin group who had functioning grafts were switched to conventional immunosuppression because of nephrotoxicity. These early studies established ciclosporin as a more effective immunosuppressive agent while illustrating the problem of nephrotoxicity.

The results of liver and heart transplantation were both improved following the introduction of ciclosporin, although once again renal function was found to be worse in the heart transplant patients who received ciclosporin (Starzl et al. 1981; Oyer et al. 1983; Emery et al. 1986). The availability of ciclosporin was one of the factors that enabled lung transplantation to be per-

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complex that inhibits calcineurin. They thereby prevent the dephosphorylation of NFAT and hence the expression of IL-2 and other genes. It is believed that many of the toxic non-immunosuppressive effects of the ciclosporin and tacrolimus are related to the effect of calcineurin inhibition on the function of other regulatory proteins

formed successfully (Reitz et al. 1982; Toronto Lung Transplant Group 1986).

The use of ciclosporin gradually evolved with lower dose regimens being introduced in combination with other immunosuppressive agents and the development of dose adjustment according to the concentration of ciclosporin present in the patient's blood (therapeutic drug monitoring, TDM). The aim of combination therapy was to obtain synergistic immunosuppression by using drugs that act at different stages of the T cell activation cascade but which have non-overlapping toxicities (Fig. 2) (Banner and Lyster 2003).

The introduction of tacrolimus for organ transplantation made its greatest impact in the field of liver transplantation. Absorption of the Sandimmun formulation of ciclosporin is dependent on bile flow. Early after liver transplantation, when a T-tube biliary diversion is normally in place, Sandimmun was not absorbed effectively, necessitating intravenous therapy. In contrast, oral therapy with tacrolimus can be established early after liver transplantation. Clinical trials found that the use of tacrolimus, when compared with Sandimmun, was associated with lower acute rejection rates, including corticosteroid-resistant rejection, and similar graft and patient survival (European FK506 Multicentre Liver Study Group 1994; U.S. Multicenter FK506 Liver Study 1994). In renal transplantation, tacrolimus use was again associated with lower rates of rejection (Vincenti et al. 1996; Mayer et al. 1997; Jensik 1998; Knoll and Bell 1999). In heart transplantation, a tacrolimus-based immunosuppression regimen had a similar efficacy to one based on ciclosporin but with a different profile of side effects (Taylor et al. 1999).

Based on data indicating that pharmacokinetic variability associated with the Sandimmun formulation of ciclosporin was a factor limiting efficacy (Lindholm and Kahan 1993; Kahan et al. 1996), a new formulation of ciclosporin (Neoral) was introduced. Direct comparison of the two formulations in heart transplant recipients showed similar safety together with graft and patient survival rates, while there was a reduction in the incidence of steroid-resistant rejection (Eisen et al. 2001).

Due to the very different potency and pharmacokinetic profiles of ciclosporin and tacrolimus together with the need for TDM (which necessitates 'open label' clinical trials), it has not been possible to establish a clear advantage for one agent over the other except in specific circumstances. There are, however, differences in the side effects of the two CNIs.

6.1.2 Treatment of Acute Rejection

Although CNIs are effective in the prophylaxis of rejection, alone they are inadequate for the treatment of established acute rejection. This is because a range of effector mechanisms have been initiated which are not sensitive to calcineurin inhibition (Rose and Hutchinson 2003). Nevertheless, proper maintenance of immunosuppression, including a CNI, is an essential part of treatment because it will control the T cell-mediated cytokine release. Treatment of acute rejection normally includes the use of high-dose corticosteroids with or without antithymocyte globulin or muromonab-CD3 (Banner and Lyster 2003).

6.1.3 Rescue Therapy

Many studies have examined the role of transferring patients from ciclosporin to tacrolimus as 'rescue therapy' for acute rejection that is refractory to conventional therapy. Unfortunately, the nature of the study populations and the clinical situation has precluded the use of randomised studies. Therefore, the data from such studies have been anecdotal and uncontrolled. Nevertheless there are data to support the view that switching from ciclosporin to tacrolimus can be a beneficial part of the treatment for refractory rejection (Woodle et al. 1996; Mentzer et al. 1998).

6.1.4 Hyperacute and Chron

Hyperacute and Chronic Rejection

Hyperacute rejection is caused by preformed antibodies and will not be influenced by the administration of a CNI. Acute rejection is a risk factor for chronic rejection, and so CNI-based immunosuppression may indirectly help to prevent chronic rejection; but there is little evidence that CNIs are efficacious in the treatment of established chronic rejection.

6.1.5 Current Practice

In recent years, regimens for maintenance immunosuppression after organ transplantation have usually consisted of a CNI combined with one or more other drugs. Corticosteroids are almost invariably used and are usually combined with a third agent such as azathioprine (Amenabar et al. 1998), mycophenolate (European Mycophenolate Mofetil Cooperative Study Group 1995), sirolimus (Kahan 2000) or everolimus (Eisen et al. 2003). Doses of ciclosporin and tacrolimus are normally adjusted in the light of blood concentrations, clinical response and evidence of nephrotoxicity. There are no laboratory investigations that can directly determine the appropriate degree of immunosuppression for an individual patient.

6.2 Allogeneic Bone Marrow Transplantation

Both ciclosporin and tacrolimus have a role increasing the rate of engraftment and in prophylaxis against, as well as treatment of, GVHD (Hows et al. 1982; Storb et al. 1986; Storb et al. 1988; Przepiorka et al. 1996; Arranz et al. 2002).

6.3 Indications Other than Organ Transplantation

From their mechanism of action, the CNIs might be expected to have a role in the management of autoimmune disease. However, their impact in this area has been limited. They act at the earliest stage of activation of the cognate immune system. In transplantation, where the timing of the antigenic stimulus from the allograft is known, they can be used prophylactically. However, in autoimmune disease, the initial stimulus to the immune system is obscure and the fault may lie in immune regulation rather than a response to an external trigger. By the time the disease is recognised clinically, multiple mechanisms may be involved, many of which are insensitive to the actions of CNIs. The adverse effects of the CNIs, including their nephrotoxicity, have been acceptable in the field of transplantation, where the objective is to maintain the function of a life-sustaining allograft and where there have been few alternatives. In autoimmune disease, however, the objective is usually to minimise morbidity rather than to avoid mortality, and so the acceptable level for toxicity is less. Nephrotoxicity may limit the long-term use of CNIs, and relapse of the disease is often a problem when the drug is discontinued. Furthermore, for many of autoimmune diseases there are often alternative treatments available. Generally, in the treatment of autoimmune disease the dose of CNI has been adjusted according to renal function rather than the drug concentration in blood, with the aim to emphasise the need of minimising toxicity rather than maximising efficacy. Currently, the CNIs are not first-line treatment for any autoimmune disease, but they may be of value in cases that have proved refractory to other treatment or where other therapy has caused unacceptable toxicity.

6.3.1 Nephrotic Syndrome

This clinical syndrome of oedema and hypoalbuminaemia coupled with heavy proteinuria and associated dyslipidaemia is caused by a number of renal pathologies including minimal change nephropathy, focal segmental glomerular sclerosis, membranous nephropathy and mesangial proliferative glomerulonephritis. Minimal change disease often responds well to corticosteroid with or without cyclophosphamide, but ciclosporin is also effective and can be used in cases where a remission can only be maintained with unacceptable levels of steroid treatment or where relapses are frequent (Niaudet 1994; Bargman 1999). Combination treatment using ciclosporin and corticosteroids can be of value in focal segmental glomerulonephritis (Cattran et al. 1999). There are few data on the role of ciclosporin in treating nephrotic syndrome of other causes (Muirhead 1999).

6.3.2 Rheumatoid Arthritis

Rheumatoid arthritis is a serious chronic systemic inflammatory disorder characterised by a deforming symmetrical erosive polyarthritis that may be associated with a number of extra-articular complications which include vasculitis, interstitial pneumonitis, mononeuritis multiplex and Sjögren's syndrome. Treatment involves the use of anti-inflammatory drugs plus disease-modifying agents and corticosteroids. Ciclosporin is effective but is regarded as a second-line agent because of the potential for renal toxicity (Berg et al. 1986; Dougados et al. 1988; Forre 1994; Panayi and Tugwell 1997). Tacrolimus also has a disease-modifying effect (Furst et al. 2002). In refractory cases, ciclosporin has been used in combination with other disease-modifying drugs (Tugwell et al. 1995; van den Borne et al. 1998).

6.3.3 Dermatological Disorders

CNIs are also effective in treating a number of dermatological disorders that are mediated by the cellular components of the adaptive immune system. Systemically administered ciclosporin has been used in the treatment of psoriasis (Ruzicka 1996) and atopic dermatitis (Sowden et al. 1991). However, long-term use is limited by nephrotoxicity. Systemically administered tacrolimus is effective in the treatment of psoriasis with a similar risk of nephrotoxicity (Jegasothy et al. 1992; The European FK 506 Multicentre Psoriasis Study Group 1996).

Tacrolimus and an analogue, pimecrolimus, penetrate the skin and are available as topical formulations (Marsland and Griffiths 2002; Reynolds and Al-Daraji 2002). Topical tacrolimus is effective in the treatment of atopic eczema (Nghiem et al. 2002), but is not effective in chronic plaque psoriasis (Zonneveld et al. 1998), possibly as a result of insufficient skin penetration. The systemic absorption of topically administered tacrolimus or pimecrolimus is low and the levels observed are considerably lower than those used in transplantation.

6.3.4 Other Conditions

Ciclosporin has been investigated as a treatment for a number of other autoimmune conditions where it has not been found to have a major role either because of limited efficacy or nephrotoxicity. Ciclosporin is active against uveitis (Nussenblatt et al. 1985) but because of problems with nephrotoxicity (Palestine et al. 1986; Austin et al. 1989) it is usually reserved for cases which are refractory to other therapy or severe bilateral sight-threatening uveitis (Walton et al. 1998).

Type 1 diabetes is an autoimmune disease. Ciclosporin has been found to increase the frequency and duration of early remission of disease but has not been found to alter the long-term outcome or need for insulin therapy (The Canadian-European Randomized Control Trial Group 1988).

Ciclosporin has been used in the treatment of acute severe ulcerative colitis that has failed to respond to high-dose corticosteroid therapy. However, these patients are usually referred for colectomy because remission may only be short term and because of the risk of nephrotoxicity (McCormack et al. 2002). Ciclosporin may have a role as a corticosteroid-sparing agent in patients with chronic ulcerative colitis (Actis et al. 1999).

7 Toxicity

7.1 General Effect of Immunosuppression

Pharmacological immunosuppression is not specific for the response to the transplanted organ or the disease under treatment. Therefore patients receiving immunosuppressive treatment are at increased risk of infection and of certain types of malignancy (Fishman and Rubin 1998; Penn 2000; Euvrard et al. 2003). Calcineurin-inhibitors principally affect the adaptive immune response of T cells and leave the innate immune system intact. Therefore conditions such as chronic viral infection that are particularly controlled by T cell responses would be expected to be increased in patients receiving CNIs (Cohen 2000; Banner 2003). However, because CNIs are used in combination with other immunosuppressive agents, their selective effect on the immune system is lost and a wide spectrum of infections may be encountered (Kahan 1989; Fishman and Rubin 1998).

7.2 Specific Toxicity

Although the calcineurin-inhibitors are potent immunosuppressive agents, their clinical use is limited by their toxicity. The commonest serious and dose-limiting problem is nephrotoxicity. A large number of other side effects can also occur. There are some differences between the toxicity of ciclosporin and tacrolimus (Table 2).

	Ciclosporin	Tacrolimus
Nephrotoxicity	++	++
Hypertension	++	+
Neurotoxicity	+	++
Glucose intolerance and diabetes mellitus	+	++
Hyperlipidaemia	++	+
Gingival hypertrophy	++	_
Hypertrichosis	+	_
Alopecia	_	+
Hyperuricaemia and gout	+	+
Hyperkalaemia and hypomagnesaemia	+	+
Liver dysfunction	+/-	+/-
Gastrointestinal symptoms	+	+
Pancreatitis	+	+
Haemolytic-uraemic syndrome/TTP	+	+
Gynaecomastia	+	+

Table 2 Comparison of the toxicity of ciclosporin and tacrolimus

Reproduced with permission from Banner and Lyster (2003).

7.3 Nephrotoxicity

The toxic effect of the CNIs on the kidney is well known, although the mechanisms underlying it are not completely understood (Wilkinson and Cohen 1999). It had been aptly described as the Achilles' heal of this form of therapy (Bennett et al. 1996). Data from kidney transplantation are difficult to interpret because the function of the renal allograft is influenced both by alloimmune injury and drug toxicity. Additionally, the renal allograft may be somewhat protected by virtue of the sympathetic denervation that accompanies transplantation. CNI nephrotoxicity has been clearly recognised after heart, liver and lung transplantation, as well as in patients being treated for autoimmune disease (Zaltzman et al. 1992; Falkenhain et al. 1996). Toxicity occurs in an acute, potentially reversible, and a chronic irreversible form. Less commonly, the kidney can be affected by the thrombotic microangiopathy. The mechanisms underlying the nephrotoxicity appear to be closely linked to the inhibition of calcineurin and are therefore similar for ciclosporin and tacrolimus.

7.3.1 Acute Toxicity

This syndrome is associated with renal vasoconstriction and functional changes that are potentially reversible. The mechanisms involved include alterations in endothelial function and in the regulation of vasomotor tone. Vasoconstriction leads to a reduction in glomerular filtration rate, salt and water retention and associated hypertension. Experimentally, endothelin appears to play a role in the vasoconstriction (Kon et al. 1995). Additionally, there is increased activity in the renin angiotensin system with increased numbers of granules in the juxtaglomerular apparatus and in hilar arterioles as well as increased expression of angiotensin type 1 receptors (Tufro-McReddie et al. 1993). Ciclosporin inhibits endothelial nitric oxide production (Andoh et al. 1997; Assis et al. 1997), endothelium-dependent vasodilation and the release of prostacyclin (Bossaller et al. 1989). Furthermore, it increases sympathetic-mediated vasoconstriction (Scherrer et al. 1990).

7.3.2 Chronic Toxicity

Chronic toxicity is associated with structural changes in the kidney. In native kidneys there is global and segmental glomerular sclerosis. Surviving glomeruli tend to be larger, indicating hyperfiltration, and proteinuria is common (Myers et al. 1988; Bertani et al. 1991). The changes are believed to be secondary to afferent arteriolar vasculopathy leading to glomerular ischaemia and tubular atrophy. The tubular atrophy and fibrosis follow a striped pattern which begins in the medullar and progresses into the medullary rays of the cortex (Palestine et al. 1986; Andoh and Bennett 1998) (Fig. 5). This anatomy means that the extent of fibrosis is often underestimated by a percutaneous core renal biopsy.

During the early phase of the disease, the correlation between the degree of structural damage and functional changes is poor (Myers et al. 1988; Ruggenenti et al. 1994; Hartmann et al. 1996). Renal function, measured by creatinine and creatinine clearance, may be stable, but structural changes may be occurring with progressive tubulointerstitial injury. This dissociation between functional and structural changes can also be demonstrated in animal models (Elzinga et al. 1993).

The renin angiotensin system appears to play a role in renal fibrosis (Kon et al. 1995; Shihab et al. 1996). Ciclosporin increases the expression of transforming growth factor- β and may thereby increase the fibrotic response to injury (Khanna et al. 1997). Tacrolimus may not have this effect (Mohamed

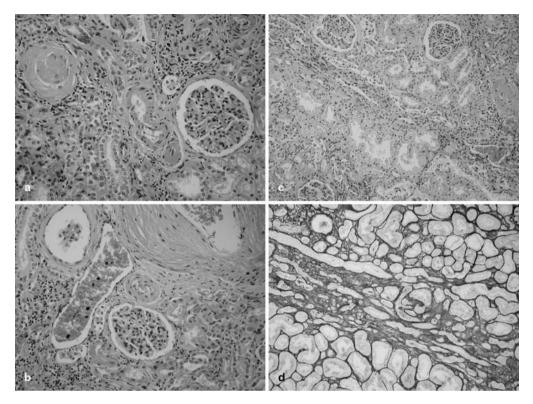


Fig. 5a–d Renal histopathological changes of chronic ciclosporin nephropathy. **a** Hyaline arteriolosclerosis, focal interstitial nephritis, glomerulosclerosis and focal tubular atrophy. **b** Intimal sclerosis of an artery, interstitial nephritis, hyaline arteriolosclerosis. **c** Focal tubular atrophy with striped fibrosis, glomerulosclerosis and hypertrophied tubules to either side of the zone of atrophy. **d** Striped fibrosis: atrophied tubules enmeshed in dense reticulin (corresponding to the zone of striped fibrosis in panel **c**). Staining: Grocott's silver methenamine. (Photomicrographs courtesy of Dr. Margaret Burke)

et al. 2000). It has therefore been argued that tacrolimus might be associated with less nephrotoxicity than ciclosporin; however, this has not been established in clinical studies, and another study failed to confirm this mechanistic difference between the two agents (Khanna et al. 1999).

7.3.3 Thrombotic Microangiopathy

The two uncommon related thrombotic microangiopathic (TMA) syndromes, haemolytic-uraemic syndrome (HUS) and thrombotic thrombocytopoenic purpura (TTP), are associated with infection, drug toxicity and pregnancy, and occur in familial and idiopathic forms. Both have been asso-

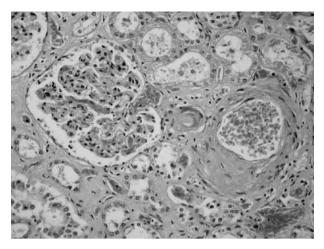


Fig. 6 Renal histopathological changes associated with the haemolytic–uraemic syndrome. Fibrinoid necrosis in an afferent arteriole and hyaline intimal thickening of an artery. (Photomicrograph courtesy of Dr. Margaret Burke)

ciated with the use of calcineurin inhibitors. The clinical features of HUS are a non-immune (Coombs' test-negative) microangiopathic haemolytic anaemia, acute renal failure and thrombocytopaenia associated with microvascular thrombosis (Fig. 6). TTP shares these features, although the degree of renal involvement is variable, but there is also neurological involvement with changing symptoms and signs. There may be a fever in either HUS or TTP. The pathophysiology of both conditions is believed to be injury to the endothelium and its consequent dysfunction (Kulzer and Wanner 1998).

CNI-associated TMA has mainly been observed in renal transplantation but has also occurred after other types of organ and bone-marrow transplantation (Ichihashi et al. 1992; Verburgh et al. 1996; Young et al. 1996). The risk may be increased by high levels of drug exposure and in some cases a cofactor such as infection can be demonstrated. An idiosyncratic susceptibility may be involved, but cases have been described where the CNI has been successfully used again once the TMA has resolved (Guella et al. 1998).

7.3.4 Management

Acute nephrotoxicity is dose-related and normally reversible by either dose reduction or, where possible, withdrawal of the CNI.

Chronic toxicity is more difficult to detect at an early stage because of the poor correlation between functional and structural changes. The use of lower doses of CNIs (combining them with other immunosuppressive drugs) and the use of dose adjustments based on the measurement of blood levels has led to some reduction in toxicity, but the effect has not been dramatic and the problem remains unsolved (Myers et al. 1988). This may be partly because of the limitations of therapeutic drug monitoring (see below), but is also because of the close link between the calcineurin inhibition that is necessary for immunosuppression and the nephrotoxicity. This means that there is no true 'therapeutic range' where efficacy can be achieved while simultaneously avoiding toxicity. Furthermore, it appears that patients vary in their susceptibility to nephrotoxicity, although the basis of this variation is currently unknown.

Tight control of blood pressure is important in slowing any progression, as is the treatment of proteinuria, usually by use of an angiotensin-converting enzyme inhibitor or a receptor blocker (Joint National Committee 1997; Mackenzie and Brenner 1998). CNI dose reduction, and when feasible, withdrawal may be of value. However, this increases the risk of allograft rejection. Additionally, some patients with established chronic nephrotoxicity may show a progressive fall in renal function despite withdrawal of the drug (Myers et al. 1988; Zietse et al. 1994). Those who develop end-stage renal failure need renal replacement therapy and will subsequently require renal transplantation (Kuo et al. 1995).

The treatment of CNI-associated thrombotic microangiopathy consists of withdrawing the drug, plasma exchange (Bell et al. 1991; Onundarson et al. 1992) and supportive therapy, including renal replacement therapy if necessary. The role of anticoagulation and antiplatelet agents is unproved. The prognosis of CNI-associated TMA is uncertain because information about this syndrome is based on case reports and no large case series has been published.

7.4

Hypertension

Hypertension is common after all forms of organ transplantation and this is related to the use of CNIs. The mechanisms linking the CNIs to hypertension are not fully understood but, as previously described, they interfere with vascular control mechanisms and increase activity of the sympathetic nervous system. In addition, the nephrotoxicity caused by these drugs may play a role in the development of hypertension (Andoh et al. 2001; Johnson et al. 2002). Interestingly, tacrolimus appears to be less potent in inducing hypertension than ciclosporin, although the reason for this remains unknown (Taylor et al. 1999).

7.5 Dyslipidaemia

Dyslipidaemia is common in organ transplant recipients. Its prevalence is partly due to the frequent use of corticosteroids, but the CNIs also play a role (Ballantyne et al. 1989; Kasiske et al. 1991). Once again, tacrolimus has a less adverse effect than ciclosporin (Taylor et al. 1999). Treatment of hypercholesterolemia must take into account the interaction between the CNIs and statins (Ballantyne 2000; Christians et al. 2002).

7.6 Diabetes Mellitus

Diabetes mellitus is another common complication of organ transplantation that is related to corticosteroid use. The CNIs are a further contributory factor (Roth et al. 1989). There is evidence that the risk is greater with tacrolimus (Pirsch et al. 1997), although this finding has not been confirmed in all studies (Taylor et al. 1999).

7.7 Neurotoxicity

Acute neurotoxicity is an infrequent but severe complication of CNI use. The syndrome consists of headache, visual impairment (including cortical blindness), generalised tonic-clonic seizures, encephalopathy and coma; less frequently dysphasia and cerebellar symptoms can occur. Paraesthesia and a burning sensation in hands and feet occasionally occur. Headache, visual disturbance and seizures are the most common manifestations. The syndrome occurs within days or weeks of starting CNI treatment. It is more common in children and adolescents than in adults and is also commoner after liver and bone marrow transplantation than after other types of organ transplant. Additional features that are sometimes present include hypertension, hypomagnesaemia and hypocholesterolaemia (de Groen et al. 1987; Jarosz et al. 1997; Derici et al. 2001). Hypocholesterolaemia and activity of the P-glycoprotein transport system may affect the concentration of the CNI present within the central nervous system (CNS) (de Groen et al. 1987; Sugawara et al. 1990; Schinkel et al. 1995). The differential diagnosis includes other CNS drug toxicity, opportunistic CNS infection (meningitis and encephalitis) and TTP. Typical findings on magnetic resonance imaging (MRI) are cerebral oedema within the cortex and juxtacortical white matter of the posterior cerebral hemispheres (Schwartz et al. 1995; Coley et al. 1999). The pathogenesis of the condition is not fully understood but seems to be related to CNI vascular toxicity. The MRI findings closely resemble those seen in hypertensive encephalopathy (Schwartz et al. 1995). The oedema appears to be vasogenic in nature rather than reflecting ischaemia (Coley et al. 1999). Treatment involves control of any hypertension, reduction in the dose and blood level of the CNI, together with supportive measures, including the use of analgesics and anticonvulsants as necessary. The clinical and MRI findings are usually completely reversible, although irreversible injury has been reported (Esterl et al. 1996). Recurrence of this acute syndrome during long-term therapy is unusual.

Chronic neurological side effects of the CNIs include headaches, which are often migrainous in nature, and tremor (Plosker and Foster 2000). The headaches can occasionally can be severe but usually respond to conventional therapy (Maghrabi and Bohlega 1998). Hand tremor can be troublesome in some patients but usually improves as the dose of CNI is reduced with increasing time after transplantation.

7.8 Other Side Effects

These are summarised in Table 2 and have been described in detail elsewhere (Kahan 1989; Plosker and Foster 2000). Cosmetic side effects of ciclosporin which can be troublesome for women (hirsutism) and children (hirsutism and coarsening of the facial features during growth) are absent with tacrolimus. In addition, the troublesome gingival hypertrophy which happens in some patients on ciclosporin does not occur with tacrolimus.

8 Pharmacokinetics and Drug Interactions

Both ciclosporin and tacrolimus are substrates for the cytochrome P450 (CP450) family of enzymes and P-glycoprotein (Pgp) transport system. This leads to a complex set of pharmacokinetic considerations that are important for the effective clinical use of the CNIs (Fig. 7). CP450 and Pgp underlie many of the drug interactions that occur between the CNIs and other therapeutic agents (Christians et al. 2002).

An orally administered CNI is subject to countertransport by P-glycoprotein and metabolism by CP450 3A within the wall of the small intestine (Lin et al. 1999). Variation in the activity of these two systems results in differences between individuals in the oral bioavailability of the CNIs (Ptachcinski et al. 1986; Shimada et al. 1994). Furthermore, the administration of drugs or other substances that alter Pgp and CP450 activity can radically change bioavailability of the CNIs (Christians et al. 2002). Following absorption, the CNI is subject to further 'first pass' metabolism by CP450 in the liver before reaching the systemic circulation (Karanam et al. 1994; Bekersky et al. 2001). The resulting metabolites are primarily excreted in the bile, although

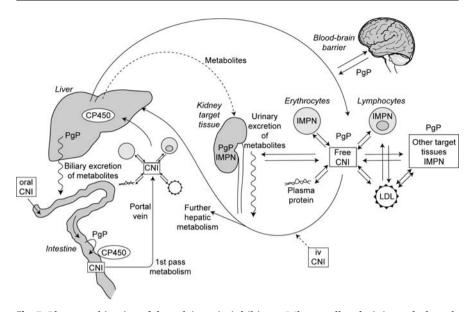


Fig. 7 Pharmacokinetics of the calcineurin inhibitors. When orally administered, the calcineurin inhibitors (*CNIs*) ciclosporin and tacrolimus are subject to countertransport by P-glycoprotein (*Pgp*) and metabolism by cytochrome P450 (*CP450*) 3A in the wall of the intestine. Absorbed drug passes via the portal circulation to the liver where further metabolism occurs. The majority of CNI metabolites are excreted via the bile. In health, only a small fraction of metabolised during recirculation through the liver. In the blood, only a small fraction of these drugs exist in the free state; most is bound to cells (both drugs) and plasma proteins (tacrolimus) or lipoproteins including low-density lipoprotein or LDL (ciclosporin). Free drug appears to enter cells via diffusion although, in the case of ciclosporin, entry may also be facilitated by lipoproteins. Intracellular drug is subject to countertransport by Pgp. The drug concentration at the intracellular site of action. Intravenously administered drug (*iv CNI*) bypasses 'first pass' metabolism in the intestine and liver so that the dose required intravenously will be lower than the oral dose

a small proportion will also enter the circulation. This proportion will increase in the face of hepatic dysfunction (Bekersky et al. 2001). Although the CNIs are metabolised in the liver, dose adjustment may be needed if a patient develops renal failure; this is probably because uraemia can interfere with hepatic metabolism by down-regulation of hepatic CP450 (Pichette and Leblond 2003).

When administered intravenously, the CNI will avoid 'first pass' metabolism in the intestine and liver and directly enter the systemic circulation. Consequently, the intravenous dose required to achieve any blood concentration is considerably lower than the oral dose (Banner and Lyster 2003). For the same reason, intersubject variation is reduced during intravenous administration. Drug elimination from the systemic circulation is principally via hepatic metabolism.

Only a very small proportion of CNI is unbound in the blood. The majority is associated with its binding proteins within red cells or bound to plasma proteins (in the case of tacrolimus) or lipoproteins (in the case of ciclosporin) (de Groen 1988; Nagase, Iwasaki et al. 1994). Therefore, the total concentration of CNI in blood that is measured during therapeutic drug monitoring (TDM) will be influenced by the haematocrit and the concentration of proteins and lipoproteins in the blood. Additionally, activity of the P-glycoprotein transporter will determine the relationship of the intra- to extracellular drug concentration (Chaudhary et al. 1992). Thus, there is no simple and predictable relationship between the measured concentration and that present at the drug's site of action (Sugawara et al. 1990; Sandborn et al. 1995; Schinkel et al. 1995). Further details of the pharmacokinetics of the CNIs are available elsewhere (Ptachcinski et al. 1985; Kahan 1989; Venkataramanan et al. 1995; Christians et al. 2002).

Most pharmacokinetic drug interactions involving the CNIs are based on either induction or inhibition of CP450 3A (Banner and Lyster 2003). However it is now becoming clear that alterations in Pgp activity contribute to these effects and, by altering the distribution of drug within compartments, Pgp can alter the drug's immunosuppressive and toxic effect independently from the total concentration measured in blood (Christians et al. 2002). Recognised interactions where other drugs alter the metabolism of the CNIs are summarised in Table 3; interactions where the CNIs make an important alteration to the metabolism of other drugs are listed in Table 4. Pharmacodynamic interactions also occur where the toxicity of other drugs are additive or synergistic with those of the CNIs. Since nephrotoxicity is a frequent problem during therapy with the CNIs, other drugs with a potential for nephrotoxicity are a particular problem, e.g. non-steroidal antiinflammatory agents (Sheiner et al. 1994), amphotericin (Kennedy et al. 1983) and foscarnet (Morales et al. 1995). Physicians prescribing for patients being treated with CNIs should be cognisant of such interactions and be able to anticipate their potential effect. Close observation and dose adjustment with the help of TDM is required to prevent an unnecessary loss of efficacy or toxicity (Banner and Lyster 2003). Pharmacodynamic interactions must be assessed by clinical observation and laboratory surveillance of the function of the relevant organ (e.g. serum creatinine and creatinine clearance in the case of nephrotoxicity).

Factors that influence the distribution of the drug will alter the relationship between the total concentration measured in the blood and the concentration at the site of action within the cell. Drugs that affect the P-glycoprotein transport system may influence this equilibrium and, thus, could alter the drug's concentration at its intracellular site of action, thereby modulating both immunosuppressive efficacy and toxicity (Sugawara et al. 1990; Table 3 Some important pharmacokinetic interactions affecting the bioavailability of ciclosporin and/or tacrolimus

Drugs that increase CNI metabolism and reduce bioavailability			
Rifampicin (rifampin)	Furlan et al. 1995; Hebert et al. 1999		
Anticonvulsants			
Phenytoin	Keown et al. 1984		
Phenobarbitone	Carstensen et al. 1986		
Carbamazepine	Cooney et al. 1995		
Prednisolone	Undre and Schafer 1998		
St John's Wort	Barone et al. 2000		

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Drugs that reduce CNI metabolism and increase bioavailability

Imidazole antifungal agents Fluconazole Assan et al. 1994; Manez et al. 1994 Itraconazole Banerjee et al. 2001 Ketoconazole Keogh et al. 1995; Floren et al. 1997 Clotrimazole Mieles et al. 1991 Macrolide antibiotics Erythromycin Ptachcinski et al. 1985; Furlan et al. 1995 Clarithromycin Wolter et al. 1994; Sadaba et al. 1998 Sirolimus (effect on tacrolimus only) Lampen et al. 1995; Lampen et al. 1998 Chloramphenicol Schulman et al. 1998 Calcium channel blockers Verapamil Robson et al. 1988 Diltiazem Valantine et al. 1992; Hebert and Lam 1999 Methylprednisolone Klintmalm and Sawe 1984 Theophylline Boubenider et al. 2000 HIV protease inhibitors Sheikh et al. 1999; Schvarcz et al. 2000 Danazol Shapiro et al. 1993; Passfall et al. 1994 Grapefruit juice Edwards et al. 1999

Table 4 Pharmacokinetic interactions where the CNIs make an important change to the metabolism of another drug

Reduced metabolism and increased drug effect			
HMG-CoA reductase inhibitors (statins)			
Lovastatin	Norman et al. 1988; Olbricht et al. 1997		
Simvastatin	Weise and Possidente 2000		
Atorvastatin	Jacobsen et al. 2000		
TOR inhibitors			
Sirolimus	Christians and Sewing 1993; Kaplan et al. 1998; Lampen et al. 1998		
Everolimus	Crowe et al. 1999; Jacobsen et al. 2001		
Divergent effects			
Mycophenolate/mycophenolic acid			
Metabolism increased by ciclosporin	Smak Gregoor et al. 1999		
Metabolism decreased/unchanged	Zucker et al. 1997; Christians et al. 2002		
by tacrolimus			

Chaudhary et al. 1992; Schinkel et al. 1995). This may be one of the mechanisms underlying the increase in ciclosporin nephrotoxicity that has been observed when it has been used in combination with sirolimus or everolimus (Andoh et al. 1996; Kahan 2000; MacDonald 2001; Eisen et al. 2003). However, this explanation remains a hypothesis, and one study conducted in primates failed to demonstrate an altered distribution of ciclosporin when it was co-administered with everolimus (Serkova et al. 2000).

9 Therapeutic Drug Monitoring

Methods of monitoring therapy with CNIs have become an established part of clinical practice. The reasons for this include: the potential for serious toxicity with CNIs, the importance of maintaining immunosuppressive efficacy to protect life-sustaining organ transplants, the wide range of variation between individuals in bioavailability and metabolism together with the large number of drug interactions between CNIs and other agents (Banner and Lyster 2003). Guidelines have been developed for the monitoring of ciclosporin and tacrolimus (Jusko et al. 1995; Oellerich et al. 1995).

The concentrations measured are those in whole blood and are only indirectly related to those at the site of action (Holt et al. 1994; Oellerich et al. 1998). Consequently, the TDM results must be interpreted in the light of other information including efficacy (lack of acute rejection) and toxicity (particularly renal). A 'therapeutic range' derived for a population of patients is usually wide and individuals with drug levels within the range may still experience rejection, nephrotoxicity or opportunistic infection. The appropriate therapeutic range will be determined by a series of factors including the time after transplantation and the other immunosuppressive agents being used (Banner and Lyster 2003). The choice of a specific 'target range' for an individual patient remains a matter of clinical judgement which must be based on the patient's individual response.

'Trough levels' are usually measured just before the administration of the next dose. In the case of tacrolimus, such levels appear to be a good measure of systemic exposure to the drug (Jusko et al. 1995). However, the correlation of trough level to exposure is less good in the case of the Neoral formulation of ciclosporin, and alternatives, such as the blood concentration 2 h after an oral dose, appear to be better measures (Levy 2001).

10 Conclusion

The discovery of the CNIs was one of the most significant advances in clinical organ transplantation. Ciclosporin improved the results of renal transplantation and transformed other types of organ transplantation from experimental to standard therapy. Both the newer agent tacrolimus and the reformulation of ciclosporin, Neoral, have advantages over the original Sandimmun formulation. The problem of nephrotoxicity remains a factor that limits the use of these drugs outside the field of transplantation. Furthermore, chronic renal failure has become one of the long-term complications of organ transplantation. Attempts to ameliorate this problem through careful drug monitoring and by using reduced doses of these drugs in combination with other immunosuppressive agents have only been partially successful. New approaches to long-term immunosuppressive treatment are needed and alternative drugs are becoming available. The potent immunosuppressive effect of the CNIs means that they are likely to continue to play a role in the initial period after transplantation when the risk of rejection is greatest, but it seems likely that they may be replaced with other drugs in the long-term maintenance treatment of patients with allografts.

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Targeted Therapy with Imatinib: An Exception or a Rule?

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Abstract The discovery of STI571, a drug targeting the tyrosine kinase activity of Bcr-Abl, c-Kit, and platelet-derived growth factor receptor (PDGFR), has demonstrated the feasibility of ATP-competitive small-molecule kinase inhibitors for the chronic treatment of molecularly defined cancers. The presence of an activated form of protein kinases targeted by STI571 in various malignancies appears to be mandatory for a clinical response to this drug. This also indicates that a certain subset of molecularly defined tumors depends upon the overactivation of one or more signaling pathways, which can be attacked by targeted therapy. The finding that STI571 resistance is often associated with mutations in the kinase domain unambiguously demonstrates that the targets of STI571 in these tumors are Bcr-Abl, c-Kit, and/or PDGFR. Generation of resistance to STI571 is most likely due to the binding mode of STI571 that stabilizes the inactive conformation of the target kinases. Recent mutational analysis combined with structural biology has provided the basis for the understanding of the binding mode of STI571, and the resistance mechanisms as well as activation mechanisms of Bcr-Abl, c-Kit, and PDGFR. Although this binding mode is key for the exquisite selectivity and tolerability of STI571, it is also its "weakness", because it allows mutations to occur in the target kinase which appear not to affect the normal function of the kinase. Therefore, for an effective long-term cancer therapy with ATP-competitive small-molecule kinase inhibitors, it appears necessary to use more than one kinase inhibitor targeting different conformations of the same kinase, or each inhibitor needs to be aimed at a specific mutant and downstream signaling element.

Keywords Protein kinase inhibitors · STI571 · Gleevec · CML · Abl kinase · c-Kit · PDGFR

Abbreviations	
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
Bcr-Abl	Fusion protein arising from the breakpoint cluster region gene (BCR)
	fusing with the Abelson (ABL) gene
CML	Chronic myeloid leukemia
GIST	Gastrointestinal stromal tumor
CMML	Chronic myelomonocytic leukemia
DFSP	Dermatofibrosarcoma protuberans
HES	Hypereosinophilic syndrome
IC ₅₀	Concentration causing 50% inhibition
Kit	Receptor of stem cell factor
PAP	Phenyl-aminopyrimidine
PDGF	Platelet-derived growth factor
PDGFR	Receptor of platelet-derived growth factor
РКС	Protein kinase C family of kinase subtypes involved in mitogenic
	signaling and metabolic pathways
PTK	Protein tyrosine kinase
RPTK	Receptor PTK

1 Introduction

The increased understanding of signal transduction pathways in normal and neoplastic cells has provided new strategies for therapeutic intervention in cancer (Hanahan and Weinberg 2000). Among the various signaling molecules, protein kinases have become an important group of drug targets, as these enzymes play a central role in diverse biological processes such as cell growth, differentiation, and apoptosis (Pawson and Nash 2000; Schlessinger 2000; Blume-Jensen and Hunter 2001; Manning et al. 2002). The recent development of specific kinase inhibitors blocking the activity of deregulated kinases appears to be sufficient to inhibit growth of tumors in vitro, in vivo, as well as in the clinic (Levitzki 1996; McMahon et al. 1998; Strawn and Shawver 1998; Matter 2001; Traxler et al. 2001).

The approach to design protein kinase inhibitors directed to the ATP binding site suffers from two major obstacles: access to the intracellular targets and selectivity. As there are more than 500 kinase in the human ge-

nome, the generation of inhibitors with an absolute specificity is unlikely to be achieved (Manning et al. 2002). The main emphasis, therefore, should be on inhibitors possessing a "reasonable" selectivity profile along with an "acceptable" side effect profile.

The recent discovery and development of STI571 (imatinib mesylate; Glivec; Glevec; CGP57148), which was approved by the FDA on 10 May 2001 for the treatment of chronic myelogenous leukaemia (CML), shows that targeting the ATP binding of protein kinases for the treatment of various diseases is feasible.

Based upon its clear disease association in CML, the protein tyrosine kinase of Bcr-Abl, represents an ideal drug target to validate the clinical utility of protein kinase inhibitors as therapeutic agents (Daley et al. 1990; Gishizky et al. 1993; Gishizky 1996; Li et al. 1999). In fact, STI571 showed potent in vitro and in vivo antitumor activity in preclinical models that were selective not only for Bcr-Abl, but also for c-Kit and platelet-derived-growth factor receptor (PDGFR) kinases (Druker et al. 1996; Traxler et al 2001; Manley et al. 2002). Initially, clinical trials in CML and acute lymphoblastic leukaemia (ALL) demonstrated that STI571 is well tolerated and showed promising clinical responses (Druker et al. 2001; Kantarjian et al. 2002a,b; Sawyers et al. 2002; Talpaz et al. 2002; O'Brien et al. 2003). Later, clinical responses were reported for STI571 in diseases with deregulated PDGFR activity, like hypereosinophilic syndrome (HES) (Gleich et al. 2002; Cools et al. 2003a,b; Griffin et al. 2003), systemic mastocytosis (Pardanani et al. 2003), gastrointestinal stromal tumors (GIST) (Joensuu et al. 2001; Tuveson et al. 2001; VanOosterom et al. 2001; Heinrich et al. 2002, 2003a) and chronic myelogenous monocytic leukaemia (CMML) (Golub et al. 1994; Ross et al. 1998; Sjöblom et al. 1998; Magnusson et al. 2001; Schwaller et al. 2001; Steer and Cross 2002) or with deregulated c-Kit activity like GIST and/or in acute myelogenous leukaemia (AML) (Heinrich et al. 2002). Although STI571 showed impressive responses in chronic phase of CML, GIST, and HES, many advanced-stage patients develop drug resistance during treatment and subsequently relapse. Although the reasons for STI571 resistance in CML are multiple, mutations in the kinase domain appear to be the predominant mechanism (reviewed in Cowan-Jacob et al. 2004). Such resistance mechanisms might prove to be an issue for protein kinase inhibitors directed towards genetically unstable cells, and therefore a good understanding of this phenomenon is important for the development of new cancer therapies.

2 Development of STI571

Almost two decades ago, when protein kinases were only available as more or less well-characterized biochemical activities obtained following purification from various tissues, there were some ideas in the pharmaceutical industry that protein kinases could represent potential oncology targets. In 1985 at Ciba-Geigy (now Novartis), one medicinal chemistry project was focusing on protein kinase C (PKC). PKC was believed to be involved in tumor promotion and growth modulation, due to the fact that it represented at that time the major receptor for tumor-promoting phorbol esters (Asaoka et al. 1992; Dekker and Parker 1994). The first "lead compound" that was identified to effectively inhibit PKC was the natural compound staurosporine, which was believed, at that time, to be a PKC-specific inhibitor (Tamaoki et al. 1986). A medicinal chemistry program was subsequently initiated around the staurosporine scaffold, which yielded the more selective PKC inhibitor CGP41251 (PKC412; midostaurin) (Fabbro et al. 1999, 2000). Following several iterative rounds of derivatization and optimization on the staurosporines, it was decided to switch to a new scaffold directed towards the ATP-binding site of PKC. Soon a phenyl-amino pyrimidine (PAP) derivative was discovered which had very promising "lead-like" properties and a high potential for chemical derivatization. This PAP derivative, with a 3'-pyridyl group (Fig. 1, element 1), showed potent enzyme and cellular activity against PKC (Zimmermann et al. 1996a,b, 1997). The introduction of a "flag-methyl" group (Fig. 1, element 2) ortho to the anilino-amino group, then led to a socalled control PAP derivative devoid of PKC inhibitory activity. Most importantly, this "control PAP derivative" revealed protein tyrosine kinase activity against Abl, c-Kit, and PDGFR (Zimmermann et al. 1996a,b, 1997, 2001; Buchdunger 2000). It was rapidly recognized that the presence of an amide group on the phenyl ring provided potent inhibitory activity against these tyrosine kinases (Fig. 1, element 3). However, the first series of these Abl, c-Kit, and PDGFR inhibitors showed poor oral bioavailability and low solubility, which was improved by addition of a highly polar, N-methylpiper-

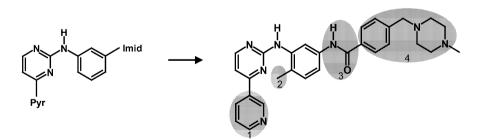


Fig. 1 Lead optimization: from PAP to STI571. An ATP-competitive lead from a smart screen (*left*) inhibited PKC, Abl, and PDGFR at IC_{50} of 1 μ M, >10 μ M, and >10 μ M, respectively. Lead optimization led to STI571 (*right*), an ATP-competitive compound with the core structure of the lead compound, but enhanced cellular activity (1), switch of selectivity (loss of PKC inhibition) but inhibition of Abl, PDGFR, and Kit (2), potency against tyrosine protein kinases (3), and increased solubility and oral bioavailability (4)

azine side chain (Fig. 1, element 4). All of these PAP modifications resulted in the methyl piperazine derivative, originally coded CGP57148 and thereafter named STI571 (signal transduction inhibitor 571), imatinib mesylate, Glivec, or Gleevec (Zimmermann et al. 1996a,b, 1997, 2001; Buchdunger 2000).

3 Pharmacological Profile of STI571

3.1 Bcr-Abl (CML)

The hallmark of CML is the Philadelphia (Ph) chromosome, which is detected in virtually all cases of CML and in 20%–30% of cases of all adult ALL (Khouri et al. 1995; Cortes et al. 1996; Gishizky 1996; Warmuth et al. 1999). The majority of patients with CML and a significant fraction of Ph chromosome positive (Ph⁺) ALL have one of the three different versions of this Bcr-Abl fusion protein (p185, p210, or p230 kDa), with additional rare cases of chromosomal translocation occurring where the Abl kinase (or Arg, a related Abl gene) (Okuda et al. 2001) comes under the control of yet other proteins (Khouri et al. 1995; Cortes et al. 1996; Gishizky 1996; Warmuth et al. 1999).

In vitro studies using purified enzymes showed that STI571 is an ATPcompetitive inhibitor of Abl, Arg, c-Kit, and PDGFR, but is devoid of significant activity against most other kinases (Druker et al. 1996; Traxler et al. 2001; Zimmermann et al. 2001; Fabbro et al. 2002; Manley et al. 2002; Cowan-Jacob et al. 2004). This selectivity profile was also observed in cellular systems, where STI571 showed potent inhibition of autophosphorylation of p210Bcr-Abl (Druker et al. 1996; Carroll et al. 1997), p185Bcr-Abl, and Tel-Abl (Carroll et al.1997), as well as activated c-Kit or PDGFR, with these activities correlating with the inhibition of cell proliferation (Buchdunger et al. 1996, 2000; Carroll et al.1997; Gambacorti-Passerini et al. 1997; Beran et al. 1998; Dan et al. 1998; le Coutre et al. 1999; Heinrich et al. 2000; Fabbro et al. 2002). Bcr-Abl activates multiple signaling pathways, including the Ras-Raf-ERK, JAK-STAT, PI3 K, JNK/SAPK, NF-KB, and c-myc, which are inhibited by STI571 (Sillaber et al. 2000; Sattler and Griffin 2003). STI571 selectively induced apoptosis that is dependent on Stat5-induced up-regulation of Bcl-xL in Bcr-Abl-positive cell lines and primary leukemia cells from Ph⁺ CML as well as ALL without affecting Ph chromosome-negative (Ph⁻) cells (Horita et al. 2000). In addition, STI571 inhibited the colony formation from peripheral blood and bone marrow from Ph⁺ CML patients, with a 92%-98% decrease in Bcr-Abl-positive colonies with little effect on normal hematopoiesis (Carroll et al. 1997; Deininger et al. 1997; GambacortiPasserini et al. 1997; Beran et al. 1998; Dan et al. 1998; Kasper et al. 1999; le Coutre et al. 1999). STI571 also prevented down-regulation of p27 levels in Bcr-Abl expressing cells as this oncogene has been shown to promote cell cycle progression by activating cyclin-dependent kinases (cdk) and down-regulating the cdk inhibitor p27 (Gesbert et al. 2000; Jonuleit et al. 2000).

The finding that expression of Bcr-Abl in mice induces a disease resembling CML provided strong evidence that the Bcr-Abl oncoprotein was one of the major driving factors in the pathophysiology of CML (Daley et al. 1990; Gishizky et al. 1993; Li et al. 1999). The in vitro antiproliferative activity of STI571 was confirmed in animal models using Bcr-Abl-transformed 32D cells in syngeneic mice, while it showed no antitumor activity against tumors derived from v-src transformed 32D cells, compatible with the selectivity profile of the compound (Druker et al. 1996). The in vivo activity of STI571 against Bcr-Abl-driven tumors was confirmed and extended to models employing cells derived from CML patients in blast crisis (Druker et al. 1996; Deininger et al. 1997; Kasper et al. 1999). Oral treatment with STI571 led to the inhibition of p210Bcr-Abl tyrosine phosphorylation correlating with tumor-free survival of the animals, suggesting that a continuous exposure of STI571 was required for optimal anti-leukemic effects (Buchdunger et al. 1996; Druker et al. 1996). STI571 has also been shown to have good oral activity in a murine bone marrow transplantation model using retroviral p210bcr-abl transduction (Wolff and Ilaria 2001).

CML has a triphasic clinical course: a chronic phase, an accelerated phase, and a terminal blast crisis resembling acute leukemia in which myeloid or lymphoblast precursors fail to differentiate. The chronic phase of CML more closely resembles a myeloproliferative disorder rather than a leukemia, as during this phase of the disease the terminal differentiation of cells is normal (Tenen 2003). The defined pathogenic events and the chronic phase of CML, which represents an almost unique "hyperproliferative disease that depends solely on the Bcr-Abl oncoprotein" was key for the impressive clinical responses induced by STI571 in this limited set of cancer patients.

Since the initial phase I dose-escalation study with STI571 in June 1998 with patients that failed therapy with interferon (IFN)- α , the responses in chronic-phase CML to STI571 have been quite durable, although most patients with blast-phase CML or Ph⁺ALL have developed resistance to STI571 and have relapsed (Druker et al. 2001; Kantarjian et al. 2002ab; Sawyers et al. 2002; Talpaz et al. 2002; O'Brien et al. 2003). The reasons for this resistance and/or relapse appear to be multiple, but data from recent clinical trials show that half of the relapsed patients carry point mutations in the kinase domain of Bcr-Abl. More than 30 mutations have been identified so far in the Abl kinase domain that renders this enzyme less sensitive to STI571 (reviewed in Cowan-Jacob et al. 2004).

3.2 PDGFR

In addition to inhibiting Bcr-Abl, Abl, Arg, and c-Kit, STI571 also inhibits the PTK of the PDGFR. PDGF has important roles in the regulation of growth and differentiation of various mesenchymal cell types during embryonal development. In the adult, PDGF stimulates wound healing and also regulates the homeostasis of the connective tissue compartment (Heldin et al. 2002). Overactivation of PDGF signaling has been implicated in several disorders, including tumors, atherosclerosis, and fibrotic conditions.

3.2.1

Chronic Myelomonocytic Leukemia

As a consequence of a reciprocal t(5;12) chromosomal translocation, the trans-membrane and the intracellular domains of the PDGFR β gene are fused to various partners like TEL (ETV6) (Golub et al. 1994), H4/D10S170 (Schwaller et al. 2001), HIP1(Ross et al. 1998) and RAB5 (Magnusson et al. 2001) all of which encode putative oligomerization domains. The resulting constitutive activation of the PDGFR β kinase generates a myeloproliferative disorder which is characterized by eosinophilic leukemia or CMML (Steer and Cross 2002), a subtype of the myelodysplastic syndrome which may progress to an acute leukemia. STI571 not only inhibited the in vitro proliferation of cells transformed by these oncoproteins (Beran et al. 1998; Sjöblom et al. 1998) but has also displayed antitumor activity in vivo in a bone marrow transplantation model driven by TEL-PDGFR β (Tomasson et al. 1999). STI571 treatment led to a normalization of eosinophils and peripheral blood cells in CMML patients involving PDGFR β translocations (Apperley et al. 2002; Magnusson et al. 2002).

3.2.2

Hypereosinophilic Syndrome

HES is characterized by persistent eosinophilia that can result in damage to various organs (Weller and Bubley 1994). Clinical studies with STI571 have reported responses in HES patients that were associated with normalization of eosinophil counts (Gleich et al. 2002, 2003). About 50% of the HES patients with durable responses to STI571 were found to have a FIP1L1-PDGFR α fusion oncogene which harbors a constitutively activated PTK activity (Cools et al. 2003a; Griffin et al. 2003). This oncogenic FIP1L1-PDGFR α was also found in patients with systemic mastocytosis that have been found to respond to the STI571 treatment (Pardanani et al. 2003). Other genetic abnormalities resulting in the activation of a "putative STI571 sensitive protein kinase" are likely to be responsible for the responses observed in FIP1L1-PDGFR α -negative HES patients that have been treated with STI571. Interestingly, one HES patient with FIP1L1-PDGFR α which relapsed

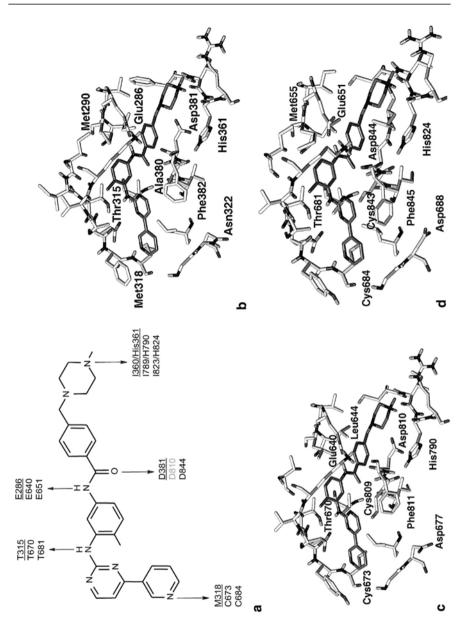


Fig. 2a–d STI571 binding to Abl, Kit, and PDGFR β . **a** Hydrogen bonds between STI571 and Abl, Kit, or PDGFR β . Hydrogen bonds between STI571 and the key residues in Abl, Kit, and PDGFR β involve: anilino-NH of STI571 with *T315* of Abl, T670 of Kit, or T681 of PDGFR β ; amide-NH of STI571 with *E286* of Abl, E640 of Kit, or E651 in PDGFR β ; amide-carbonyl of STI571 with *D381* of Abl, not to D810 of Kit, but possibly to D844 of PDGFR β (D of the DFG motif N-terminal A-loop); pyridine N of STI571 with *I360/H361* of Abl, C673 of Kit, or C684 in PDGFR β ; *N*-methylpiperazine of STI571 with *I360/H361* of Abl, I789/H790 of Kit, or I823/H824 in PDGFR β . **b** STI571 binding to Abl. Details of the

under STI571 treatment was found to contain a T674I point mutation in the kinase domain of the PDGFR α , which corresponded to a STI571-resistant mutation (T315I) found in Bcr-Abl (Cools et al. 2003b; Fig. 2). The STI571-resistant FIP1L1-PDGFR α -T674I mutant was sensitive to another PDGFR inhibitor, PKC412, which targets a different conformation of the PDGFR α kinase domain compared to STI571 (Cools et al. 2003b).

3.2.3 Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) is a locally invasive soft tissue tumor of the dermis which is characterized by translocations involving chromosomes 17 and 22 resulting in the fusion of the collagen type I α 1 chain gene (Col) with PDGF-B chain (Mandahl et al. 1990; Craver et al. 1995; Naeem et al. 1995; Pedeutour et al. 1996; Simon et al. 1997). Expression of Col-PDGF-B in fibroblasts induced PDGFR β autophosphorylation, morphological transformation, and in vivo tumor growth. Tumor growth of primary DFSP was strongly reduced by STI571 treatment, and the increase in PDGFR β tyrosine phosphorylation, due to expression of Col-PDGF-B in primary cultures of human DFSP, was decreased by treatment with STI571 (Sjöblom et al. 2001). STI571 had a significant impact with respect to tumor growth and metastatic spread in patients with DFSP (Maki et al. 2002; Rubin et al. 2002).

In summary, these early clinical trials obtained with patients suffering from DFSP, CMML, and HES show the utility of STI571 in tumors that are driven by overactivation of PDGF signaling.

3.2.4 Glioblastomas and Sarcomas

Several observations provide a rationale for clinical studies with STI571 in the treatment of glioblastomas and sarcomas:

1. PDGFR α and PDGF-A chain are co-expressed in tumor cells with increasing frequency in high-grade, as compared to low-grade, tumors (Hermanson et al. 1996).

binding of STI571 (*dark*) to Abl kinase domain (*light*). Hydrogen bonds to (*from left to right*) M318, T315, E286, D381, I360, and H361. The inhibitor has packing interactions with Phe317, Phe382, Val256, Tyr253, Ile313, Lys271, Met290, Val299, Ala380, and Val289. c STI571 binding to Kit. Details of the binding of STI571 (*dark*) to Kit kinase domain (*light*). Hydrogen bonds to (*from left to right*) C673, T670, E640, I789, and H790. d STI571 binding to PDGFR β . Details of the binding of STI571 (*dark*) to PDGFR β kinase domain (*light*). Potential hydrogen bonds to (*from left to right*) C684, T681, E651, D844, I823, and H824

- 2. Amplified and oncogenic forms of the PDGFR α gene have been found in glioblastomas (Fleming et al. 1992; Kumabe et al. 1992; Clarke and Dirks 2003).
- 3. Expression of PDGF-B and PDGFR β are frequently observed in various soft tissue tumors and sarcomas (Smits et al. 1992), while in Ewing family sarcomas the expression of PDGF-C is strongly up regulated (Zwerner and May 2001).
- 4. Experimental glioblastoma can be inhibited in in vivo animal models by STI571 (Shamah et al. 1993; Strawn et al. 1994; Kilic et al. 2000), while dominant-negative PDGF-C, as well as AG1296, a PDGFR kinase antagonist, inhibited growth of Ewing sarcoma cells in vitro (Zwerner and May 2002).

As mutations causing constitutive activation of PDGFR α have recently been found in a subset of GISTs that were responsive to STI571 (Heinrich et al. 2003a,b), it cannot be excluded that the PDGFR may be mutated more frequently in tumors, including glioblastoma and sarcomas. The utility of PDGF signaling inhibitors like STI571 for the treatment of sarcoma and glioblastoma will only be revealed in carefully controlled clinical trials.

3.2.5

PDGF Signaling in Non-oncological Indications

The rationale for PDGF signaling antagonists in non-oncological indications, such as vasculoproliferative disease, is linked to the upregulation of PDGF signaling in smooth muscle cells derived from atherosclerotic vessels, as well as from neointima lesions (Ross et al. 1990; Tanizawa et al. 1996; Ueda et al. 1996; Rubin et al. 1998). In animal models of restenosis, significant efficacy has been obtained either with neutralizing antibodies to PDGF or with different types of inhibitory approaches (Ferns et al. 1991; Myllarniemi et al. 1997; Sirois et al. 1997; Banai et al. 1998; Bilder et al. 1999; Giese et al. 1999; Hart et al. 1999; Yamasaki et al. 2001; Bilder et al. 2003). Although these studies are encouraging and have indicated that PDGF antagonists might be useful clinically for the treatment of vasculoproliferative diseases, there are some concerns as to whether inhibition of PDGF signaling alone will be sufficiently efficacious for the treatment atherosclerotic lesions that usually involve multiple pathways and cells (Sihvola et al. 1999; Kozaki et al. 2002; Sihvola 2003).

Reduced cell proliferation, collagen synthesis, and deposition have been observed in bleomycin-induced fibrosis with PDGFR antagonists like AG1296 or by expressing a soluble form of the PDGFR β (Rice et al. 1999; Yoshida et al. 1999). Treatment of glomerulonephritis with either STI571 or PDGF aptamers led to a significant reduction in mesangial cell proliferation and collagen deposition preventing renal scarring, suggesting a therapeutic potential of PDGF antagonists in fibrotic conditions (Floege and Ostendorf 2001).

Last but not least, stimulation of PDGF signaling was found to contribute to increased interstitial fluid pressure (IFP) in experimental tumors (Pietras et al. 2001, 2002, 2003). Therefore, inhibition of PDGF signaling, with STI571 or other antagonists, may be useful in lowering the intra-tumoral fluid pressure, facilitating an increased uptake of cytotoxic drugs in tumors that result in an improved therapeutic efficacy.

3.3 c-Kit

3.3.1 STI571 in GIST

Deregulation of c-Kit has been implicated in the etiology of a number of cancers, including AML, small cell lung cancers, gliomas, testicular cancer, and GIST. The majority of the GIST patients carry mutations in c-Kit that result in constitutively enhanced ligand-independent PTK activity, which appears to play a key role in the pathogenesis of these tumors (Hirota et al. 1998; Lux et al. 2000; Demetri et al. 2002; Heinrich et al. 2002; Joensuu et al. 2002). Since these Kit mutations lead to uncontrolled cell proliferation via permanent stimulation of downstream signaling pathways, it was thought that GIST should prove sensitive to STI571. Although the activity of STI571 against c-Kit was initially of concern, due to the important role that this receptor appears to play in hematopoiesis, the c-Kit-activating mutations associated with GIST supported the use of STI571 in the treatment of this tumor, which is refractory to chemotherapy and radiation. The remarkable clinical activity of STI571 led to its approval in February 2002 for the treatment of advanced GISTs.

Clinical responses in GIST patients following treatment with STI571 appear to be closely associated with the presence of activating c-Kit mutations, as patients expressing wild-type Kit had a significantly lower response (Heinrich et al. 2003a,b). In the subset of patients that expressed wild-type c-Kit and responded to STI571 treatment, activating mutations in the PDGFR α have been found. These clinical trials demonstrated for the first time the presence of naturally occurring mutated forms of PDGFR α . The mutational status of PDGFR α could explain response and sensitivity to imatinib in GISTs lacking c-Kit mutations, because mutations in c-Kit and in PDGFR α appear to be mutually exclusive.

Although STI571 is an effective therapy in GIST with an impressive response rate of 62% after 15 months of treatment (Demetri et al. 2002; Joensuu et al. 2002), relapses have been observed in up to 20% of patients. It seems very likely that one of the underlying mechanisms for this STI571 re-

sistance will be the emergence of clones carrying point-mutations in the kinase domain of c-Kit, in close analogy to the resistance induced in Bcr-Abl by STI571 in CML.

3.3.2 STI571 Binding to c-Kit and PDGFR

An understanding of the molecular interactions at the atomic level between STI571 and Abl was key to understand the binding mode between STI571 and the inactive conformation of c-Kit (Manley et al. 2002). Six hydrogenbonding interactions between STI571 and c-Kit, which are found in the STI571-Abl structure, were suggested by homology modeling, and five of these were recently confirmed by analysis of the crystal structure of a complex between c-Kit and STI571 (Jacob et al. unpublished) (Fig. 2). The predicted amide-C=O interaction with D810 of the DFG motif does not occur due to a very slight twist of STI571 in binding to c-Kit compared to Abl. Assuming a similar auto-inhibitory conformation as in Abl and c-Kit, these H-bonds are also believed to be important interactions between STI571 and PDGFR β (in PDGFR α the residues interacting with STI571 are identical) (Fig. 2).

The interactions comprise:

- 1. Anilino-NH with T670 (T315 in Abl and T681 in PDGFR β).
- 2. Amide-NH with E640 (E286 in Abl and E651 in PDGFR β).
- 3. Amide-C=O with D810 of the DFG motif does not occur in c-Kit (occurs in Abl with D381, and possibly in PDGFR β with D844).
- 4. Pyridine-N with C673 (M318 in Abl and C684 in PDGFR β).
- 5. *N*-methylpiperazine with I360 and H361 (I789/H790in Abl and I823/H824 in PDGFR β).

Interestingly, STI571 is inactive against the activating mutations found in systemic mastocytosis, seminoma, and AML that affect position D816 (Heinrich et al. 2002). Mutations in D816, which lies four amino acids beyond the DFG motif in the A-loop, appear to disfavor the auto-inhibitory conformation of the A-loop such that STI571 does not bind.

4 Resistance to STI571

4.1 Kinase Domain Mutations

STI571 resistance may be caused by up-regulation of the Bcr-Abl, by STI571 efflux mechanisms, and/or plasma binding to α -1 acidic glycoprotein

(Gambacorti-Passerini et al. 2000, 2002, 2003; Mahon et al. 2000; Weisberg and Griffin 2000; Hughes et al. 2002; Jørgensen et al. 2002; le Coutre et al. 2003). However, the predominant mechanism of resistance appears to be due to mutations in the kinase domain of Bcr-Abl, which renders the kinase less sensitive to STI571 inhibition. More than 30 single missense mutations have been found in relapsed CML patients (Kuwazuru et al. 1990; Griffin 2000; Mahon et al. 2000; Gambacorti-Passerini et al. 2000, 2002, 2003; Weisberg and Griffin 2000; Barthe et al. 2001; Gorre et al. 2001a,b; Hochhaus et al. 2001, 2002, 2003; Al-Ali et al. 2002; Branford et al. 2002, 2003; Hofmann et al. 2002a; Hughes et al. 2002; Kreil et al. 2002; le Coutre et al. 2002; Leguay et al. 2002; Roche-Lestienne et al. 2002; von Bubnoff et al. 2002; Hochhaus 2003), some patients possess multiple cell populations with different Bcr-Abl mutations, and at least one patient has been observed to have two mutations in the same Bcr-Abl molecule (Shah et al. 2002).

Structural studies have provided evidence for the binding mode of STI571 to Abl and have revealed how point mutations identified in relapsed patients can lead to resistance (Schindler et al. 2000; Hantschel et al. 2003; Nagar et al. 2002, 2003; Cowan-Jacob et al. 2004). The structure of the catalytic domain of Abl complexed to STI571 (or a close analog thereof) showed that the Abl-kinase adopts a so-called auto-inhibited or inactive conformation (Xu et al.1999; Schindler et al. 2000; Manley et al. 2002; Nagar et al. 2002, 2003; Hantschel et al. 2003). The main feature of this binding mode is that the A-loop, which controls the catalytic activity of kinases, adopts a self-inhibitory conformation by imitating the bound substrate. This binding mode probably accounts for the relatively high selectivity of STI571, but at the same time allows mutations to occur which lower the affinity to STI571 without affecting the kinase activity or ATP-binding affinities (Shah et al. 2002; Cowan-Jacob et al. 2004).

Although various mutations display varying degrees of STI571 resistance, the affinities for ATP for the various mutant forms of recombinant Abl were similar to the wt-Abl kinase (Shah et al. 2002; Cowan-Jacob et al. 2004). The most common mutants in Ph⁺ leukemias are E255K/V, T315I, and M351T, with G250E, Q252H/R, Y253H/F, F317L, and E355G being less frequent, while mutants most resistant to STI571 are T315I, G250E, E255K and Y253H. The mutations appear to pre-exist and may not to occur due to drug exposure (Shah et al. 2002; Branford et al. 2003). While most of the mutations that lead to STI571 resistance result in the destabilization of the auto-inhibited conformation of the Abl kinase domain, the Y253F/H, T315I, F317L, F359V, and F382L mutations directly affect the binding to STI571 (Shah et al. 2002; Cowan-Jacob et al. 2004). The mutations cluster in the following regions of the Abl kinase (Fig. 3):

- *The hinge region*. It connects the N-terminal with the C-terminal lobe and harbors the T315I mutant, which is one of the most resistant to STI571. This

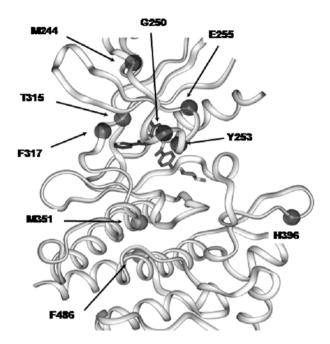


Fig. 3 STI571-resistant mutations in the Abl kinase domain. Locations of the main STI571-resistant mutations on the structure of Abl kinase in complex with STI571s. The IC₅₀ (*in parentheses*) concentrations were determined in vitro with the recombinant versions of the Abl kinase that was used for crystallization: wtAbl (0.18 uM); single mutations in the ATP binding (T315I: >10 uM; F317L: 0.40 uM); G-loop (E255K: >10 uM; Y253H: >10 uM; G250E: 1.6 uM; E258G: 1.3 uM); A-loop (H369P: 0.90 uM); Distant Muts (M244 V: 0.68 μ M; M351T: 0.30 μ M; F486S: 0.87 μ M; M244V: 0.68 μ M)

change leads to a steric clash between the isoleucine side chain and STI571, together with the loss of an H-bond to the drug. A mutation of the analogous T674I gatekeeper residue has also been identified in the PDGFR α kinase of an HES patient who relapsed under STI571 treatment (Cools et al. 2003b), and is also likely to occur in c-Kit driven malignancies (T670I) (Fig. 2). In contrast to T315I, the other hinge region mutation in Abl, the F317L, causes a relatively minor disturbance in the STI571 binding (Shah et al. 2002; Cowan-Jacob et al. 2004).

- The G-loop (P-loop). It usually adopts a β-sheet conformation in the active Abl kinases; whereas when STI571 is bound to the inactive form, the G-loop forms a cage around the pyridine-pyrimidine rings of STI571, stabilized by numerous interactions to other parts of the kinase. Any mutations disrupting one of these interactions, or favoring other G-loop conformations will result in STI571 resistance. It should be pointed out that mutations within the G-loop may also lead to an enhanced activation of the kinase (increased oncogenicity) that prevents STI571 to "freeze" the inactive conformation of Abl.

- A-loop. Mutations in the activation segment, such as H396P or H396R, show weak resistance to STI571 (Hochhaus et al. 2002; Shah et al. 2002; von Bubnoff et al. 2002) resulting from the loss of an H-bond interaction between P396 and N414 (Cowan-Jacob et al. 2004). In contrast, the reasons for the loosening of STI571 binding of mutations clustered on the N-terminal part of the A-loop (V379I, F382L and L387M) can be quite diverse and have been discussed recently (Shah et al. 2002; Cowan-Jacob et al. 2004).
- N-terminal lobe. The M244 V and F311I mutations are located in the N-terminal lobe, which is a region that seems capable of adapting to the binding of different inhibitors (Al-Ali et al. 2002; Hochhaus et al. 2002; Kreil et al. 2002; Roche-Lestienne et al. 2002; Shah et al. 2002). Exactly how these mutations cause resistance is still poorly understood (Cowan-Jacob et al. 2004).
- C-terminal lobe. Another group of mutations is located in the C-terminal lobe, close to the piperazinyl group of STI571. Except for F359V, none of the other mutations (E355G, M351T, S417Y, E459 K, F486S) are in direct contact with STI571. The most frequently occurring mutant M351T displays only a moderate resistance to STI571 (Al-Ali et al. 2002; Branford et al. 2002; Hochhaus et al. 2002; Kreil et al. 2002; Roche-Lestienne et al. 2002; Shah et al. 2002). The mutation E459K at the bottom of the C-terminal lobe is very remote from the STI571 binding site but in close proximity to the myristoyl binding site of Abl (Hantschel et al. 2003; Nagar et al. 2003). It is conceivable that this mutation has a direct effect on destabilizing the assembled inactive state of Abl, therefore indirectly favoring an activated conformation of the kinase domain to which STI571 has a lower affinity. The inactive state of the "full-length" Abl has a similar arrangement of the SH3, SH2, and kinase domains as in Src, although the manner by which Src and Abl are kept in a catalytically inactive state differ, particularly with respect to helix C (Hantschel et al. 2003; Nagar et al. 2003; Xu et al. 1997, 1999).

Additional mutants outside of the kinase domain of Bcr-Abl will probably be identified in patients relapsing following STI571 treatment. As shown by an elegant in vitro random mutagenesis study, about 90 mutations were found in Bcr-Abl that imparted resistance to STI571, without affecting the transforming ability of Bcr-Abl (Azam et al. 2003). One third of all of these mutants were outside of the kinase domain and probably cause an allosteric destabilization of the auto-inhibited conformation by involving residues that participate in interactions between the kinase domain and the regulatory N-terminal cap, SH3 and SH2 domains (Azam et al. 2003).

4.2 Overcoming STI571 Resistance

While the kinase domain mutations that still retain some STI571 sensitivity (IC50<3 uM) may be overcome with more potent inhibitors that interact with the STI571 binding conformation, the resistance caused by mutants in the G-loop or the T315I mutant may require inhibitors that bind to Abl in an alternative mode or target the same conformation without engaging in an H-bond with the gatekeeper residue T315. Targeting the active, ATP-binding conformation of Abl has the advantage that the likelihood of emergence of resistant mutants is reduced. Prototype compounds in this regard are the pyrido[2,3-d]pyrimidine derivatives, one of which has been shown to bind to the catalytically active conformation of the Abl kinases, although the DFG motif is in a conformation that is unsuitable for optimal binding of ATP (Nagar et al. 2002). In addition, this pyrido[2,3-d]pyrimidine derivative has been reported to inhibit various mutants of the G-loop, the M351T mutant, as well as the A-loop mutant H396P, but not T315I (Dorsey et al. 2000; La Rosée et al. 2002; Cowan-Jacob et al. 2004). However, because the conformation of activated protein kinases is highly conserved, a major disadvantage of these types of inhibitors is the lack of selectivity against other kinases which might limit their tolerability (Kraker et al. 2000).

In contrast to the highly conserved conformations of activated protein kinases, the inactive states of protein kinases often adopt conformations in which there are large variations in size and shape of the ATP-binding pocket (Huse and Kuriyan 2002). Although targeting the inactive conformation of kinases may result in a better selectivity profile, the caveat is that these inhibitors will interact with many residues not involved in binding ATP, allowing mutations to arise which will prevent binding of the inhibitor without affecting the kinase activity.

Treatment strategies geared towards improving the STI571 therapy and/ or circumventing STI571 resistance are multiple and may include:

- 1. Inhibition of other kinases acting downstream of Bcr-Abl, such as the Src family members Lyn and Hck (Danhauser-Riedel et al. 1996; Donato et al. 2003; Warmuth et al. 2003) or Btk (Hofmann et al. 2002b)
- Inhibitors targeting other signaling molecules like Mek/Erk (Yu et al. 2002), PI3 K (Klejman et al. 2002), farnesyltransferase inhibitors (Topaly et al. 2002; Daley 2003; Hoover et al. 2002), protein degradation (Gatto et al. 2001), or Hsp90 (Gorre et al. 2002), all of which have shown to enhance the antiproliferative effects of cells expressing STI571 resistant Bcr-Abl

The most likely treatment of malignancies targeted by STI571 will be a cocktail of drugs designed to inhibit a particular class of STI571-resistant mutations or a mechanism of resistance reminiscent to the treatment of

HIV. Similar treatment strategies are likely to be applied in diseases other than CML where the drug targets the PTK of c-Kit and PDGFR (i.e., CMML, GIST, HES).

5 Conclusion and Perspectives

Although the clinical efficacy of STI571 has clearly demonstrated the utility and the potential of kinase inhibitors, the defined etiologies of diseases associated with expression of mutated PDGFR, c-Kit and Abl may make the success of this kinase inhibitor as single agent unique for a limited set of cancers. On the other hand, STI571 has taught us several lessons:

- 1. Protein kinase inhibitors with a "reasonable selectivity" are feasible and can be used for the chronic treatment of cancer patients with an acceptable tolerability and efficacy profile where the pathophysiology of the kinase target in the tumor is molecularly defined.
- 2. In almost all clinical studies with STI571, the clinical responses to drug treatment correlate with the presence of an activated form of the target kinase in the tumor. These findings indicate that at least some (subsets) of tumors may ultimately depend on the over-activation of one pathway which can be attacked using targeted therapy. To maximize the chances of success in targeted therapy it will be mandatory to stratify patients according to the relevance of the molecular target in the particular malignancy.
- 3. Although relapses under STI571 treatment may be due to various reasons, the finding that it is often associated with mutations in the kinase domain of the target enzyme unambiguously demonstrates that the target for STI571, thus far, can only be Bcr-Abl, c-Kit, and/or PDGFR.
- 4. The exquisite selectivity of STI571, which is achieved by targeting and/or inducing the "inactive conformation of the target kinase," is presumably key for the excellent tolerability to the drug but is at the same time also its "Achilles heel." The binding mode of STI571 to the target kinase allows mutations in residues that are not involved in ATP binding and consequently not deleterious to the normal function of the targeted kinase. Mutations leading to resistance are either less likely to occur, or will generate a different set of mutations if the compound targets the active rather than the inactive conformation. To optimize treatment, it appears obvious to use more than one kinase inhibitor targeting the same kinase, but different conformations.
- 5. A key to the discovery of new anti-cancer therapeutics is the selection of epidemiologically relevant, drugable protein kinase targets coupled to efficient lead optimization for potency, selectivity, efficacy, and biopharmaceutical properties. Mutational analysis combined with structural biology has

provided the understanding on the activation mechanisms of Bcr-Abl, Kit, and PDGFR as well as to its resistance to STI571.

Protein kinase inhibitors have and will continue to offer new therapies in the coming years and have started to add novel treatment modalities for cancer. Consequently, an effective long-term management of cancer is still likely to require cocktails of drugs, each aimed at a specific mutant and pathway.

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Clinical Aspects of Imatinib Therapy

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Abstract Imatinib is an inhibitor of the ABL, platelet-derived growth factor receptors, and KIT tyrosine kinases. Given the pathogenetic role of the BCR-ABL tyrosine kinase in chronic myeloid leukemia, this was the first disease selected for clinical trials with imatinib. In the clinical trials, patients in all phases of the disease responded to imatinib and experienced minimal toxicity. Responses in patients with chronic phase disease have been durable, thus far. Clinical trials with imatinib were expanded and there are now examples of malignancies driven by each of the targets of imatinib where remarkable results have been seen. The rationale for the use of imatinib in these various diseases and the clinical trial results will be reviewed.

Keywords $CML \cdot BCR-ABL \cdot Imatinib$

1 Introduction

Imatinib mesylate (Gleevec, Glivec, formerly STI571) is an inhibitor of the ABL, platelet-derived growth factor receptor (PDGFR), and KIT tyrosine ki-

nases. Preclinical data with this compound demonstrated specific killing of cells expressing the constitutively activated BCR-ABL tyrosine kinase. As *BCR-ABL* is the causative molecular abnormality of chronic myeloid leukemia (CML), this was the first disease selected for clinical trials with imatinib. In these clinical trials, rapid responses to imatinib were observed with durable responses in patients with CML in the chronic phase. A randomized study comparing imatinib to standard therapy with interferon- α for newly diagnosed patients with CML in the chronic phase demonstrated significantly improved outcomes for patients randomized to imatinib as initial therapy. Thus, imatinib has rapidly become the standard therapy for patients with newly diagnosed CML.

Other disease indications for which imatinib has shown clinical activity are based on the KIT and PDGFR inhibitory activities of this drug. Specifically, the majority of gastrointestinal stromal tumors (GISTs) have activating KIT mutations that drive the growth of these tumors, and imatinib has shown remarkable single agent activity in this disease. Interestingly, a subset of patients with GIST and wild-type KIT expression have activating mutations of the PDGFRA that are imatinib-sensitive, and patients with these tumors also respond to imatinib. Similarly, the subset of patients with chronic myelomonocytic leukemia (CMML) with EVT6-PDGFRB rearrangements have responded well to imatinib, as have patients with dermatofibrosarcoma protuberans (DFSP), due to the activation of the PDGFRB by constitutive production of the ligand for this receptor. Lastly, patients with hypereosinophilic syndrome (HES) have recently been shown to have an intrachromosomal deletion that results in the production of an activated PDGFRA, and these patients respond extremely well to imatinib. Whether additional uses for imatinib will be found is less clear, but at a minimum, the concept of effective and selective therapies arising by targeting pathogenetic events in tumors has been validated.

2 Clinical Features of CML

CML is a clonal hematopoietic stem cell disorder. It accounts for 15%–20% of all cases of leukemia with an annual incidence of 1 to 2 cases per 100,000 per year. The median age at diagnosis is between 50 and 60 years of age. Clinically, the disease progresses through distinct phases referred to as chronic or stable, accelerated, and blast. The chronic or stable phase of the disease is characterized by excess numbers of myeloid cells that differentiate normally. After an average of 4–6 years, the disease transforms through an "accelerated phase" to an invariably fatal acute leukemia, also known as blast crisis. Disease progression is likely due to the accumulation of molecu-

lar abnormalities that lead to a progressive loss of the capacity for terminal differentiation of the leukemic clone (Faderl et al. 1999; Sawyers 1999).

Treatment choices for patients with CML prior to the introduction of imatinib included stem cell transplantation, hydroxyurea, or interferon- α -based regimens, with allogeneic stem cell transplantation being the only proven curative therapy. As the average age of onset of CML is greater than 50 years of age, this factor, plus the inability to identify a suitably matched donor limits this option to a minority of patients. Thus, fewer than 20% of CML patients were cured with these treatment options (Faderl et al. 1999; Sawyers 1999).

3 BCR-ABL as a Therapeutic Target

Virtually all patients with CML express the BCR-ABL protein. This chimeric protein is generated as a result of a reciprocal (9;22) chromosomal translocation that produces a shortened chromosome 22, the so-called Philadelphia chromosome (Ph) (Nowell and Hungerford 1960; Rowley 1973; de Klein et al. 1982; Shtivelman et al. 1985; Ben-Neriah et al. 1986). In CML patients, the BCR-ABL fusion protein has a relative molecular mass of 210 kDa. This same fusion protein is present in approximately half of adult patients with Ph-positive acute lymphoblastic leukemia (ALL). The remainder of the Ph-positive adult ALL patients and the 3% of pediatric ALL patients that are Ph positive express a smaller BCR-ABL fusion protein termed p185 or p190BCR-ABL (Chan et al. 1987; Hermans et al. 1987; Clark et al. 1988). BCR-ABL has been conclusively established as a leukemic oncogene in a variety of animal models (Daley et al. 1990; Heisterkamp et al. 1990; Kelliher et al. 1990; Huettner et al. 2000) and the transforming function of the BCR-ABL protein is dependent on the tyrosine kinase activity of the ABL portion of this protein (Lugo et al. 1990). Thus, an inhibitor of the ABL protein tyrosine kinase would be predicted to be an effective and selective therapeutic agent for these leukemias.

Imatinib is a relatively specific inhibitor of the BCR-ABL tyrosine kinase (Druker and Lydon 2000; Buchdunger et al. 2001). Pre-clinical data showed significant specific activity against *BCR-ABL*-expressing cell lines in vitro and in vivo (Druker et al. 1996). In addition, imatinib could select for the growth of *BCR-ABL*-negative hematopoietic cells from CML patient samples in colony-forming assays and long-term marrow cultures (Druker et al. 1996; Deininger et al. 1997; Kasper et al. 1999). Based on the favorable preclinical data, imatinib was advanced to clinical trials.

4 Clinical Trials in CML

4.1 Phase I and Pharmacokinetic Studies

A standard dose-escalation phase I study of imatinib began in June 1998 at three centers in the United States. The initial study was a dose escalation trial designed to establish the maximum tolerated dose with a secondary endpoint of clinical efficacy (Druker et al. 2001b). Patients with Ph-positive CML were eligible if they had failed therapy with interferon- α and were in the chronic phase of the disease, defined as less than 15% blasts in the peripheral blood and bone marrow. Patients had to be off all anti-leukemia therapy for at least 1 week prior to starting imatinib and were required to have a white blood count (WBC) of at least 20×10^9 /l. Imatinib was administered as once daily oral therapy and no other cytoreductive agents were allowed. Patients were treated continuously at their initial dose unless severe toxicity or disease progression developed.

This study enrolled 83 patients at 14 dose levels ranging from 25 to 1,000 mg. The median duration of disease at study entry was 3.8 years, and one-third of patients had signs of early progression to accelerated phase, with increased blasts or basophils in peripheral blood or marrow (Druker et al. 2001b). Imatinib was well tolerated with minimal side effects and a maximally tolerated dose could not be defined.

Pharmacokinetic studies showed that imatinib is rapidly absorbed after oral administration with a mean T_{max} of 2–4 h. The increase in mean plasma AUC was proportional to the administered dose for the dose range of 25 to 1,000 mg, although there was large interpatient variability (Peng et al. 2001). Normalizing the dose to either body weight or body surface area did not reduce interpatient variability in AUC. The mean plasma trough concentration was 0.57 µg/ml (about 1 µM) 24 h after the administration of 350 mg of imatinib at steady state, which exceeds the concentration required to inhibit proliferation of *BCR-ABL*-positive leukemic cells. The half-life of the drug is approximately 18 h (Peng et al. 2001).

Hematologic responses, defined as a 50% decrease in the WBC from baseline, maintained for at least 2 weeks, were achieved in all patients treated with 140 mg or greater of imatinib. Once doses of 300 mg or greater were reached, 53/54 patients (98%) achieved a complete hematological response (CHR), defined as a normal WBC and platelet count with no circulating immature myeloid cells, maintained for at least 4 weeks (Druker et al. 2001b). Lowering of the WBC was typically seen within the first 2–3 weeks of therapy with achievement of normal WBCs in 3–6 weeks. Normal WBCs were maintained in 51 of 53 patients with a median duration of follow-up of 310 days. At doses of imatinib of exceeding or equal to 300 mg, cytogenetic responses were seen within 5 months in 17/31 (53%) patients with 31% of patients achieving a major cytogenetic response (<35% Ph-positive) and 10% achieving a complete cytogenetic response (Druker et al. 2001b).

Given the effectiveness of imatinib in chronic-phase patients who had failed interferon, the phase I studies were expanded to include patients with CML in myeloid and lymphoid blast crisis and patients with relapsed or refractory Ph-positive ALL (Druker et al. 2001a). Patients were treated with daily doses of 300–1,000 mg of imatinib. In patients with myeloid blast crisis, 21/38 (55%) had a response to therapy, as defined by a decrease in percentage of marrow blasts to less than 15%. Seventeen of 38 (45%) cleared their marrows of blasts (<5%), with 4 of these patients (11%) meeting criteria for complete remission with full recovery of peripheral blood counts. Seven patients with myeloid blast crisis (18%) had responses lasting beyond 1 year (Druker et al. 2001a).

In patients with Ph-positive ALL or lymphoid blast crisis of CML, 14/20 (70%) responded to imatinib. Eleven of 20 (55%) completely cleared their marrow of blasts, with 4 patients (20%) meeting criteria for a complete hematologic response. All but one of the lymphoid phenotype patients relapsed between days 45 and 117 (Druker et al. 2001a).

4.2 Phase II Studies

Based on the unprecedented results in phase I studies, single agent imatinib was tested further in interferon-refractory and interferon-intolerant patients as well as in patients with accelerated phase disease and patients with CML in myeloid blast crisis and Ph-positive ALL. These studies accrued over 1,000 patients, at 30 centers in 6 countries, in 6 to 9 months. Results from these studies with 18 months of follow-up have been published (Kantarjian et al. 2002; Ottmann et al. 2002; Sawyers et al. 2002; Talpaz et al. 2002), and results updated to 30 months of follow-up are summarized in Table 1.

	Chronic phase (IFN failure)	Accelerated phase	Blast crisis
	Kantarjian et al. 2002	Talpaz et al. 2002	Sawyers et al. 2002
CHR	96%	40%	9%
MCR	64%	28%	16%
CCR	48%	20%	7%
Disease progression	13%	50%	90%

Table 1 Phase II results with imatinib

CCR, complete cytogenetic response; CHR, complete hematologic response; IFN, interferon; MCR, major cytogenetic response (Philadelphia chromosome-positive metaphases \leq 35%).

Chronic phase patients who were refractory to or intolerant of interferon- α were treated with 400 mg per day of imatinib; 454 of the 532 enrolled patients had a confirmed diagnosis of chronic-phase disease and were evaluable for response. Eligibility criteria in this study allowed inclusion of patients with up to 15% blasts and 15% basophils in the marrow or peripheral blood. Median duration of disease was 34 months and median duration of previous interferon- α therapy was 14 months. With a median follow-up of 29 months, 96% of patients achieved a CHR, with the median time to CHR being less than 1 month. Imatinib induced major cytogenetic responses in 64% of patients, with a complete cytogenetic response rate of 48%. The estimated progression-free survival at 24 months was 87% (Kantarjian et al. 2002).

Cytogenetic responses have been durable and correlate with improved progression-free and overall survival. Thus, once a patient achieves a major cytogenetic response, it is estimated that 24 months later, 91% of these patients will not have progressed. Achievement of a major cytogenetic response at 3, 6, or within 12 months was associated with a statistically significant improvement in overall survival. For example, if patients achieve a major cytogenetic response within 12 months, the estimated survival at 24 months is 99% as compared to 86% for patients with less than a major cytogenetic response (p<0.001). Baseline features that independently predicted a high rate of major cytogenetic responses were the absence of blasts in the peripheral blood, a hemoglobin exceeding 12 g/dl, less than 5% blasts in the marrow, CML disease duration of less than 1 year, and a prior cytogenetic response to interferon- α (Kantarjian et al. 2002).

Results of the phase II study in patients with accelerated-phase disease were equally impressive (Talpaz et al. 2002). Accelerated phase was defined as 15%-30% blasts or more than 30% blasts plus promyelocytes in the peripheral blood or marrow, greater than 20% peripheral basophils, or a platelet count less than 100×10^9 /l, unrelated to therapy. In this study, 235 patients were enrolled. Of these, 181 had a confirmed diagnosis of accelerated phase and were analyzed for response. With follow-up of up to three years, 83% of patients showed some form of hematologic response, with 40% of patients achieving a CHR; 28% of patients achieved a major cytogenetic response, with 20% complete responses (Talpaz et al. 2002).

In this phase II study, 62 patients with a confirmed diagnosis of accelerated phase were treated with 400 mg per day of imatinib, while 119 additional patients were treated with 600 mg per day. A retrospective analysis showed that prognostic features, such as age, sex, performance status, spleen size, and hematological parameters including WBC, percentage of marrow blasts, peripheral basophils, and hemoglobin were well matched between these two groups. A significantly improved outcome for responses and survival were observed for patients treated with 600 mg per day as compared to patients treated with 400 mg. For example, using 400 mg per day, 31% of patients obtained a CHR as compared to 45% of patients treated with 600 mg per day. The rate of complete cytogenetic responses was 24% with 600 mg per day versus 13% with 400 mg per day. The estimated survival at 24 months was 44% for patients treated with 400 mg per day versus 66% for patients treated with 600 mg per day (p=0.009). Patients who obtained a CHR, maintained for at least 4 weeks, and patients with a major cytogenetic response at 3 months also had significantly improved survival. Based on these data, 600 mg per day of imatinib is the preferred dose of therapy for patients with advanced phase disease.

Results of the phase II study treating 229 patients with a confirmed diagnosis of myeloid blast crisis showed an overall response rate of 52%, with sustained hematologic responses lasting at least 4 weeks in 31% of patients. Nine percent of patients achieved a complete remission (CR=<5% blasts) with peripheral blood recovery and another 4% of patients cleared their marrows to less than 5% blasts but did not meet the criteria for CR because of persistent cytopenias. Finally, 18% of patients either returned to chronic phase or had partial responses. Major cytogenetic responses were seen in 16% of patients, with 7% having complete responses (Sawyers et al. 2002). Median survival was 6.9 months with an estimated survival of 17% at 24 months.

Baseline features predictive of prolonged survival were (1) a platelet count greater than or equal to 100×10^9 /l, (2) peripheral blood blasts less than 50%, and (3) a hemoglobin at or greater than 10 gm/dl. For patients with all three of these features, the median survival was 21 months. However, if patients had none of these features, their median survival was only 4 months. Patients with a CHR or marrow blasts less than 5% at 2 months had a median survival of over 24 months and their survival was significantly longer than patients who were either returned to chronic phase or had no response. These results with single agent imatinib compare favorably with historical controls treated with chemotherapy for myeloid blast crisis in which the median survival is approximately 3 months. However, the high relapse rates suggest that imatinib should be viewed as either a bridge to allogeneic stem cell transplantation or that patients should be enrolled in clinical trials combining imatinib with other agents.

In patients with Ph-positive ALL, 29/48 (60%) responded to single agent imatinib. Unfortunately, the duration of response was relatively short, with a median estimated time to disease progression of only 2.2 months (Ottmann et al. 2002).

4.3 Phase III Comparison of Imatinib to Interferon in Newly Diagnosed Chronic Phase Patients

A phase III randomized study, comparing imatinib at 400 mg per day with interferon- α plus cytarabine (Ara-C) in newly diagnosed patients with chronic phase CML enrolled 1,106 patients from June 2000 to January 2001. Baseline characteristics of the 553 patients randomized to each treatment were well balanced for all features evaluated, including age, WBC, Sokal and Euro scores, and time from diagnosis. With a median follow-up of 19 months, patients randomized to imatinib had statistically significant better results than patients treated with interferon- α plus Ara-C in all parameters measured (Table 2), including rates of CHR, major and complete cyto-

 Table 2
 Phase III results of imatinib versus interferon plus cytarabine for newly diagnosed chronic phase CML patients (O'Brien et al. 2003)

	Imatinib 400 mg	Interferon+Ara-C
CHR	97%	69%
MCR	87%	35%
CCR	76%	14%
Intolerance	3%	31%
Progressive disease	3%	8.5%

CCR, complete cytogenetic response; CHR, complete hematologic response; MCR, major cytogenetic response (Philadelphia chromosome-positive metaphases<35%).

Intolerance leading to discontinuation of first-line therapy. Progressive disease to accelerated phase or blast crisis. All of these differences are highly statistically significant with p<0.001.

genetic responses, tolerance of therapy, and freedom from disease progression (O'Brien et al. 2003). Despite the fact that 76% of patients randomized to imatinib achieved a complete cytogenetic response, the majority of these patients had detectable leukemia using *BCR-ABL* transcripts as analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Hughes et al. 2002). When analyzed by log reduction in *BCR-ABL* transcript levels, 39% of patients achieved at least a 3-log reduction in *BCR-ABL* levels, but only 13% and 3% achieved a 4- and 5-log reduction, respectively.

5 Mechanisms of Relapse/Resistance to Imatinib

Response rates to imatinib in chronic phase patients are quite high and thus far, responses have been durable. Response rates are also quite high in pa-

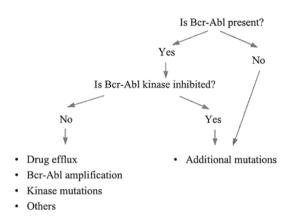


Fig. 1. Scheme for analyzing potential mechanisms of relapse

tients with advanced-phase disease, but relapses, despite continued therapy with imatinib, have been common. In all patients who have relapsed, the BCR-ABL protein remains present. A particularly useful categorization of relapsed/resistant CML patients has been to determine whether or not there is persistent inhibition of the BCR-ABL kinase (Fig. 1). Patients with persistent inhibition of the BCR-ABL kinase would be predicted to have additional molecular abnormalities besides BCR-ABL driving the growth and survival of the malignant clone. In contrast, patients with reactivation of the kinase would be postulated to have resistance mechanisms that either prevent imatinib from reaching the target or render the target insensitive to BCR-ABL. In the former category are mechanisms such as drug efflux or protein binding of imatinib. In the latter category would be mutations of the BCR-ABL kinase that render BCR-ABL insensitive to imatinib and amplification of the BCR-ABL protein.

In the largest studies of resistance or relapse, several consistent themes have emerged. In the majority of patients who respond to imatinib and then relapse while remaining on therapy, the BCR-ABL kinase has been reactivated (Gorre et al. 2001). BCR-ABL kinase activity was analyzed by assessing tyrosine phosphorylation of CRKL, a direct substrate of the BCR-ABL kinase, and the major tyrosine phosphorylated protein in CML patient samples (Oda et al. 1994; Druker et al. 2001b). In these studies, greater than 50% and perhaps as many as 90% of patients with hematologic relapse have BCR-ABL point mutations in at least 13 different amino acids scattered throughout the ABL kinase domain (Hofmann et al. 2001; Branford et al. 2002; Hochhaus et al. 2002; Roche-Lestienne et al. 2002; Shah et al. 2002; von Bubnoff et al. 2002). Some other patients have amplification of BCR-ABL at the genomic or transcript level. In contrast, in patients with primary resistance, that is, patients who do not respond to imatinib therapy, BCR-ABL-independent mechanisms are most common (Hochhaus et al. 2002).

In patients who relapse due to reactivation of the BCR-ABL kinase, the BCR-ABL kinase remains a good target. Analysis of the inhibitory activity of imatinib against these mutations has shown that some might be sensitive to dose escalation, but the most common mutation at amino acid 315 is completely insensitive to imatinib (Corbin et al. 2002). ABL kinase inhibitors with specificity that differs from imatinib have already been synthesized, and one of these compounds, PD180970, is capable of inhibiting some, but not all of the common BCR-ABL kinase mutations (La Rosee et al. 2002a). These data suggest that it may be possible to treat patients with several different ABL kinase inhibitors to circumvent resistance. Given that BCR-ABL kinase activity has been reactivated in relapsed patients, it might also be useful to target signaling pathways activated by BCR-ABL, such as RAF/ MEK/ERK, PI-3 kinase, AKT, or RAS. For example, two groups recently reported in vitro sensitivity of imatinib-resistant BCR-ABL-positive cell lines to a farnesyl transferase inhibitor (Topaly et al. 2001; Hoover et al. 2002). Moreover, Hoover et al., observed that this compound sensitized cells to imatinib, even imatinib resistant cell lines (Hoover et al. 2002). Alternatively, strategies to decrease BCR-ABL protein expression using agents such as geldanamycin, 17-AAG, or arsenic trioxide might be useful (Topaly et al. 2001; Gorre et al. 2002; La Rosee et al. 2002b).

6 Safety and Tolerability

Imatinib has generally been well tolerated, with grade 3 or 4 nonhematologic toxicities being uncommon. The most common grade 1 or 2 toxicities include fluid retention, nausea, muscle cramps, skin rashes, fatigue, and diarrhea. Myelosuppression is more common in advanced phase patients than in chronic phase patients, and imatinib induced prolonged aplasia in 1% of blast crisis patients (Sawyers et al. 2002). In contrast, patients with GIST treated with 400 or 600 mg per day of imatinib had rates of grade 3/4 neutropenia and thrombocytopenia of 5% and less than 1%, respectively, demonstrating the specificity of this side effect to leukemia patients (Demetri et al. 2002). Thus, myelosuppression is more likely to be due to suppression of the BCR-ABL-positive clone as opposed to inhibition of normal hematopoiesis. For this reason, it is not clear how much is accomplished by dose reduction for myelosuppression.

7 Gastrointestinal Stromal Tumors and KIT

GISTs are mesenchymal neoplasms that can arise from any organ in the gastrointestinal tract or from the mesentery or omentum. Although GISTs morphologically resemble leiomyosarcomas and nerve sheath tumors, they are a distinct entity (Fletcher et al. 2002). More than 90% of GISTs express KIT (Hirota et al. 1998), and biochemical evidence of KIT activation can be found in almost all GISTs (Rubin et al. 2001). In approximately 90% of cases, this activation is linked to somatic mutations of KIT, usually involving exons 9 or 11 (Rubin et al. 2001). Several lines of evidence indicate that KIT mutations are an early pathogenetic event in GISTs: (1) a similar frequency and spectrum of mutations is seen in histologically benign vs malignant tumors and early/localized vs late/metastatic tumors; (2) familial syndromes of GIST are associated with germline mutations of KIT; (3) molecular studies suggest that KIT mutations are acquired before the development of cytogenetic abnormalities (Rubin et al. 2001; Corless et al. 2002; Heinrich et al. 2002c).

The standard treatment for localized GISTs is complete resection (Blanke et al. 2001). Recurrence after complete resection is common, occurring in up to 90% of patients with larger tumors (Ng et al. 1992), and survival following recurrence is short. Patients with advanced GIST have historically done poorly, with a 30% 6-month progression-free survival following initial chemotherapy. The chemosensitivity of GISTs has been difficult to define because most published series have not adequately distinguished GISTs from leiomyosarcomas and neurogenic tumors. However, the published data suggest that the response rate of GISTs to single- or multi-agent chemotherapy is less than 5%.

The concept that patients with GISTs might benefit from treatment with imatinib was based on the above data and two additional experimental observations: (1) treatment of GIST cell lines with imatinib inhibited proliferation and induced apoptosis; (2) several GIST-associated mutant KIT isoforms were potently inhibited by imatinib in vitro at concentrations similar to wild-type KIT (Heinrich et al. 2000; Tuveson et al. 2001). Based on the identification of mutated KIT as a therapeutic target in GIST and the lack of an effective conventional medical therapy, a patient with chemotherapy-resistant gastric GIST metastatic to omentum and liver was started on imatinib at 400 mg po daily in March 2000. The tumor in this patient expressed an exon 11 mutant KIT isoform and the patient responded dramatically to imatinib therapy (Joensuu et al. 2001).

The strong pre-clinical rationale, coupled with the clinical success in this solitary case, led to the development of two proof-of-principle GIST trials utilizing imatinib. An European Organization for Research and Treatment of Cancer (EORTC), dose-escalation study of imatinib included 40 patients, 36

of whom had advanced GIST. The imatinib dosage ranged from 400 mg daily to 500 mg twice daily. At the highest dose, 5 of 8 patients had grade 3 toxicity (nausea/vomiting in 3, edema in 1, and dyspnea in 1) and 400 mg twice daily was considered the maximally tolerated dose. Of the patients, 19 (53%) had objective partial responses, while 13 (36%) had stable disease and only 4 (11%) experienced frank progression (van Oosterom et al. 2001). Two patients who progressed on 400 mg subsequently responded to 800 mg. With a minimum follow-up of 11 months, 29/36 remained on treatment. None of the four patients in the trial with a non-GIST sarcoma responded to imatinib.

The GIST Working Group (a consortium of the Oregon Health and Science University Cancer Institute, the Fox Chase Cancer Center, the University of Turku, the University of Helsinki, and the Dana-Farber Cancer Institute) performed a phase II randomized trial of 400 mg daily vs 600 mg (Demetri et al. 2002): 147 patients with incurable GIST (51% pre-treated with systemic therapy) were accrued, with a median age of 54 and median performance status of 1. Of the patients, 94% had undergone previous surgery, often involving the stomach or other areas in the GI tract; therefore, drug absorption was a concern in this trial. Nevertheless, pharmacokinetic assessments showed that drug levels were actually higher than those seen in leukemia patients given imatinib. The half-life of the drug was 20 h.

Imatinib was highly effective in this advanced disease population: 62% of patients on 400 mg daily and 65% on 600 mg achieved a partial response (Demetri et al. 2002). Stable disease was seen in 19% and 20%, respectively, and 16% and 8% had primary resistance, manifested as initial disease progression. Of the patients that failed the lower dose, 28% responded when crossed over to the higher dose. With a median follow-up of 15 months, 73% of patients remained on study drug, with an overall median time-to-treatment failure of 72 weeks.

KIT mutation analysis was performed on pre-treatment tumor samples from 121 of the 147 patients in the GIST Working Group trial (Heinrich et al. 2002b). Using a combination of PCR amplification of tumor DNA, denaturing HPLC, and direct DNA sequencing, exons 9, 11, 13, and 17 of the *KIT* gene were examined for mutations. Activating mutations were found in 86% of cases, most commonly in exon 11. Despite the fact that previous studies had suggested KIT mutations in GIST were associated with a worse prognosis (Hirota et al. 1998), patients with an exon 11 mutation had a significantly higher partial response rate (72%) than those with no detectable mutations (9%), and time-to-treatment failure was statistically significantly longer as well. Patients whose tumor harbored an exon 9 mutation did less well than those with exon 11 mutation (partial rate of 32%), but responded better and had a longer time-to-treatment failure than those with no detectable mutation. Because the numbers of patients in the exon 9 mutation group and the no mutation group were relatively small, these findings need to be confirmed. It is currently recommended that imatinib be offered to all GIST patients who are candidates for systemic therapy. KIT mutation status may be helpful in assessing the risk-benefit ratio for adjuvant therapy, and might help identify patients with advanced disease who are likely to do poorly, so that alternative agents or combination therapies can be planned.

Several additional studies of imatinib in the treatment of GIST are ongoing. The EORTC enrolled a phase II trial treating GIST patients with 400 mg BID. Phase III trials of lower vs higher dose imatinib (400 vs 800 mg daily) were enrolled by a North American Intergroup and by a European Consortium (made up of the EORTC, ISG, and AGITG), and both trials accrued rapidly.

Given the high recurrence rate for GISTs, even following complete resection, and the sensitivity of the disease to imatinib, it is reasonable to test drug administration before or following surgery in higher-risk patients. At least three trials are assessing the neoadjuvant/adjuvant use of imatinib in GIST. As there is substantial evidence that KIT mutations are pathogenetic or at least occur before other molecular abnormalities, it is predicted that imatinib would be even more active in the early disease setting and may enhance surgical results by eliminating minimal residual disease (Heinrich et al. 2002c).

Besides GIST, a number of other human cancers are associated with *KIT* mutations, including mastocytosis, seminoma, and acute myeloid leukemia (AML). Although *KIT* mutations are found in almost all cases of mastocytosis, they are found in less than 10% of cases of seminoma and are rare in adult AML (Heinrich et al. 2002a). Unfortunately, the *KIT* mutation found in mastocytosis is biochemically resistant to imatinib (Ma et al. 2002), while the sensitivity of the mutations found in AML and seminoma have not been reported. Thus, it will be necessary to develop other KIT inhibitors with activity against the *KIT* mutation found in mastocytosis.

8 PDGFR as a Therapeutic Target

Translocations involving the *PDGFRB* gene have been identified in several myeloproliferative and myelodysplastic syndromes. The most common of these translocations, (5;12)(q33;p13), is seen in a subset of patients with CMML and results in fusion of the *EVT6* (*TEL*) and *PDGFRB* genes (Golub et al. 1994). *EVT6* is a member of the ETS family of transcription factors and contains an amino terminal domain that mediates homotypic oligomerization. When fused to the membrane-spanning region of PDGFRB, this domain leads to dimerization of PDGFRB and constitutive activation of its kinase activity (Carroll et al. 1996).

Both wild-type PDGFRB and EVT6-PDGFRB are inhibited by imatinib (Carroll et al. 1997); thus, EVT6-PDGFRB represents an attractive therapeutic target. In preliminary reports, several patients with CMML containing the (5;12)(q33;p13) translocation were treated with imatinib and all achieved complete hematologic remissions (Apperley et al. 2002; Magnusson et al. 2002). This encouraging result indicates that imatinib may be used to treat tumors that are dependent on PDGFR activation. There are additional chromosomal translocations that partner the *PDGFRB* gene with *H4*, *HIP1*, *CEV14*, or *Rab5*. Affected patients are described as having a myeloproliferative disorder with eosinophilia, eosinophilic leukemia, or CMML (Schwaller et al. 2001). Though rare, all of these PDGFRB-driven myeloid proliferations are potentially treatable with imatinib.

The PDGFRB pathway is also a target in DFSP, a low-grade sarcoma of the dermis that often recurs after surgical excision. These tumors are characterized by a (17;22) translocation involving the *COL1A1* and *PDGF-B* genes, which results in over-production of fusion COL1A1-PDGF-BB ligand and consequent hyperactivation of PDGFRB (Simon et al. 1997). It has been shown that imatinib inhibits the growth of DFSP cells both in culture and in immunodeficient mice (Sjoblom et al. 2001), and preliminary results in patients look promising (Maki et al. 2002; Rubin et al. 2002).

In the GIST clinical trials, it was observed that a small subset of patients with wild-type KIT expression responded well to imatinib. Careful examination of patients with wild-type KIT-expressing tumors showed that one third of these tumors had activating mutations of the PDGFRA (Heinrich et al. 2003). These mutations occurred in two different exons. One set of mutations was imatinib-sensitive and this accounted for responses observed in patients whose tumors expressed wild-type KIT (Heinrich et al. 2003).

Another disease in which imatinib has shown remarkable clinical benefits is HES. Imatinib had initially been used empirically in this disease and the dramatic results prompted investigations of the molecular basis for imatinib's activity in this disease. Two groups independently arrived at the conclusion that an intrachromosomal deletion on chromosome 4 resulted in a fusion between a gene of unknown function, FIP1L1, and a truncated PDGFRA in a large percentage of patients with this disorder (Cools et al. 2003; Griffin et al. 2003). The resulting FIP1L1-PRGFRA fusion protein is a constitutively activated tyrosine kinase that is imatinib sensitive, thus accounting for the responsiveness of this disease to imatinib.

Both KIT and the PDGF receptors are expressed in many common tumors and have been reported to be activated by both autocrine and paracrine mechanisms. In most of these tumors, it is unclear whether monotherapy with imatinib would be useful. Most likely, KIT and PDGFR activation have a supportive rather than a pathogenetic role in these tumors. Although imatinib may have a role in the treatment of such cancers, meaningful conclusions will only be derived from carefully designed clinical trials that incorporate proteomic and genomic assessment of target activation status.

9 Conclusions

The last few years have been filled with tremendous excitement as the dramatic activity of imatinib has been demonstrated in clinical trials. The clinical development of imatinib has shown that a combination of the right drug with the appropriate target disease and patient population can reveal the true potential of such targeted agents.

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Isoquinolinesulfonamide: A Specific Inhibitor of Rho-Kinase and the Clinical Aspect of Anti-Rho-Kinase Therapy

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Abstract The light of molecular pharmacology research has been focused on protein kinase inhibitor sulfonamide derivatives since 1978. Our studies on naphthalene and isoquinoline sulfonamide derivatives over the last 25 years have achieved a pronounced contribution toward elucidating the physiological function of various protein kinases and, moreover, toward developing a new type of medicine. This chapter relates to the discovery of new protein kinase inhibitors, their mechanisms of action, and their clinical application in Japan.

Keywords Calmodulin antagonist · W-7 · Naphthalenesulfonamide ·

 $Is oquinoline sulfonamide \cdot H-7 \cdot H-8 \cdot H-9 \cdot HA1077 \cdot Rho \ kinase \ inhibitor \cdot Vasodilator \cdot Phosphorylation \ of \ MLC_{20} \cdot Vasospasm \cdot Cerebral \ is chemia \cdot Subarachnoid \ hemorrhage \cdot Cerebral \ infarction$

1 Discovery of Isoquinolinesulfonamide as a Protein Kinase Inhibitor

1.1 History of Discovery

Chemical compounds incorporating a sulfonamide residue have been synthesized for therapeutic purposes for over 70 years. In the period 1930– 1950, many compounds were patented. Prontosil was produced as an antibacterial agent, and several kinds of sulfonamide derivatives appeared as diuretic agents. Other sulfonylureas were synthesized for different indications and used clinically for diabetes mellitus. However, none of the patents on sulfonamides, as their use as medicines declined, was maintained any more in the period of 1950–1970.

In the latter half of the 1970s, Hidaka et al. brought a new and unexpected focus on certain sulfonamide derivatives (Fig. 1). In 1978, a newly synthesized compound, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) was reported to inhibit contractile response of vascular smooth muscle in concentrations ranging from 1 to 300 μ M by affecting actomyosin ATPase activity (Hidaka et al. 1978a). The molecular mechanism of W-7 action was then more thoroughly investigated (Hidaka et al. 1978b; Hidaka et al. 1979). W-7 inhibited the superprecipitation of aorta smooth muscle actomyosin, and the addition of the modulator protein calmodulin (CaM) neutralized, in a dose-dependent fashion, the inhibition of actomyosin superprecipitation by W-7 in a dose-dependent fashion.

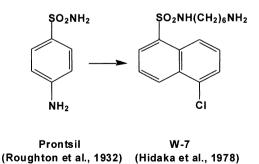


Fig. 1 Structure of sulfonamide derivatives Prontosil and W-7, anti-bacterial agent and calmodulin antagonist, respectively

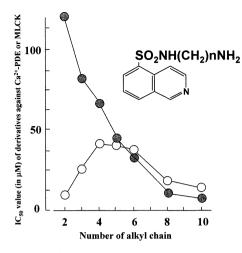


Fig. 2 Effect of naphthalenesulfonamides with various alkyl chains on calmodulin-dependent PDE and MLCK. The *closed* and *open circles* represent Ca²⁺/calmodulin-dependent PDE and MLCK, respectively

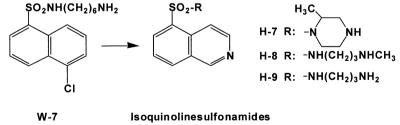
In the process of elucidating the mechanism of action of W-7, we found W-7 to bind CaM only in the presence of calcium, indicating that it inhibited the activities of CaM-dependent enzymes by preventing the binding of CaM to these enzymes (Tanaka and Hidaka 1980a). The studies indicated that W-7 was a calmodulin antagonist (Hidaka and Tanaka 1983). This was the first time that the target molecule of a sulfonamide had been clarified.

As shown in Fig. 1, W-7 has six alkyl carbon chains. We have studied the effect of W-7 and its derivatives on Ca²⁺/CaM-dependent enzymes such as Ca^{2+}/CaM -dependent cyclic nucleotide phosphodiesterase (Ca^{2+} -PDE) and myosin light chain kinase (MLCK) (Saito et al. 1987). We have synthesized several naphthalenesulfonamides with different alkyl chains, from 2-10, and examined their inhibitory effects on at least two different calcium-dependent enzymes (Fig. 2). The inhibitory activities on Ca^{2+} -PDE increased when the alkyl-carbon chain was elongated, while the inhibitory activities for MLCK did not always increase with a similar elongation of the alkyl-carbon chain. Contrasting results were clearly observed in the inhibitory activities of naphthalenesulfonamide derivatives with shorter alkyl-carbon chains (from 2-4) toward Ca²⁺-PDE and MLCK. Inhibition of Ca²⁺-PDE by ML-9, a naphthalenesulfonamide with a hexahydro-1,4-diazepine substituent corresponding to the length of two alkyl carbon chains between amino residues, was very weak, but the inhibition of MLCK by ML-9 was about ten times stronger (Saito et al. 1987). These different inhibitory potencies strongly suggested that the naphthalenesulfonamide ML-9 was a specific inhibitor of MLCK. The results also suggested that the inhibition of MLCK produced by ML-9

did not seem to be related to CaM but perhaps to a novel mechanism. Therefore, we performed the studies described below.

It is well-known that CaM-dependent enzymes partially digested by trypsin share the common property of loss of Ca²⁺-CaM dependence (Tanaka et al. 1980b). In other words, partially trypsin-digested preparations of calcium-dependent enzymes exhibit full enzymatic activity even in the absence of Ca²⁺-CaM. The removal of the inhibitory domain by proteolysis results in conversion of the enzymes into their active forms. Therefore, we prepared the Ca²⁺/CaM-independent form of MLCK by partial proteolysis in order to evaluate the direct action of the compounds toward MLCK. We obtained results similar to those shown in Fig. 2. ML-9 inhibited Ca²⁺/CaM-independent MLCK with respect to ATP with a K_i of 4.1 μ M, but it inhibited CaM action with a K_i of 50 μ M. The data indicate that ML-9 inhibits MLCK directly without interaction with CaM (Hidaka et al. 1991).

We therefore began studies to find and design new protein kinase inhibitors, considering that the compound with two alkyl chains between amino residues-which is more closely related to quinoline, isoquinoline, and quinazoline rather than naphthalene-inhibits Ca2+/CaM-independent MLCK. Following these ideas, we have succeeded in finding the structure of new protein kinase inhibitors as shown in Fig. 3 (Hidaka et al. 1984). H-9 shown in Fig. 4 was a suitable ligand for affinity chromatography. By using an H-9 affinity column, we purified various protein kinases successfully from tissues (Inagaki et al. 1985). Our results also suggested that a number of different protein kinases could be isolated through this column chromatography approach. This is very important information not only for the purification of protein kinases, but also for estimation of the relative affinities of each of the protein kinases for isoquinolinesulfonamide. As shown in Fig. 4, cyclic AMP-dependent kinase (PKA), cyclic GMP-dependent kinase (PKG), and protein kinase C (PKC) were purified by H-9 affinity column chromatography (Inagaki et al. 1985).



(calmodulin antagonist) (protein kinase inhibitor)

Fig. 3 The structure of isoquinolinesulfonamide derivatives that are protein kinase inhibitors

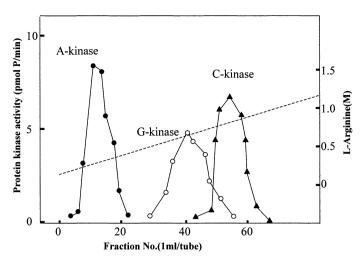


Fig. 4 H-9 affinity chromatography for purification of protein kinases. The mixed fraction, which contains PKC, PKA, and PKG partially purified from diethylaminoethyl (DEAE) column chromatography of $100,000 \times g$ supernatant of rat brain, was applied to H-9-immobilized Sepharose 4B column, and then the three protein kinases were separately eluted from the column by an l-arginine gradient

By application of this affinity column to a trial of purification of PKC, we found that we could purify PKC and we also uncovered the existence of the γ -isoform of PKC (Ohno et al. 1987). Among the inhibitor derivatives, H-7 was found to be relatively specific for protein kinase C.

1.2 Why Are Isoquinolinesulfonamides Specific for Protein Kinases?

As shown in Fig. 5, H-7 inhibited protein kinase C in competitive fashion with respect to ATP. It should be noticed that the same special arrangement of H-7 and ATP was revealed by co-crystallization of ATP or H-7 with PKA (Fig. 6) (Engh et al. 1996). This hypothesis is supported by our previous data that the isoquinolinesulfonamide H-8 inhibited all examined protein kinases competitively with respect to ATP, and the addition of H-8 protected the catalytic subunit of PKA from irreversible inactivation by the ATP analog *p*-fluorosulfonylbenzoyl-5'-adenosine (FSBA) in a dose-dependent manner. Our data (Hagiwara et al. 1987) also indicated that when the quantitative binding of H-8 to PKA was measured under conditions of thermodynamic equilibrium using a gel filtration method, the catalytic subunit bound approximately 1 mol of drug/mol of protein with apparent half-maximal binding at 1.0 μ M drug. Differences in the inhibitory potencies of the isoquinolinesulfonamide derivatives tested against various protein kinases were dependent on the stereo structure of the isoquinolinesulfonamide substituent. For example, as

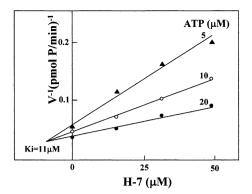


Fig. 5 Kinetic analysis of H-7-induced inhibition of PKC. The PKC reaction was performed with 20-kDa myosin light chain (MLC_{20}) as a substrate and PKC partially prepared from rabbit brain. A Dixon plot gives a K_i of 11 μ M for H-7

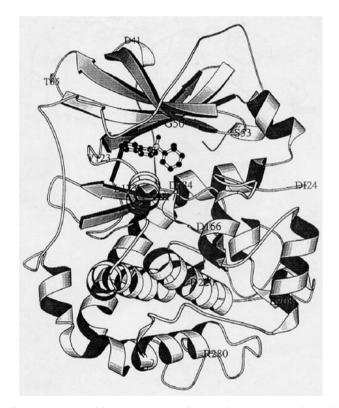


Fig. 6 Secondary structure of bovine PKA catalytic subunit in complex with H-7. The C-terminal region is displayed with DI24. (The figure was drawn by Bossemeyer and is cited with his permission)

	Compound	R	Mechanism	Pharmacological effect	
Sulfonamide deriva-	W-7	-(CH ₂) ₆ NH ₂	CaM antagonism	Vascular relaxation	<100
tives	ML-9	R1-*1	MLCK inhibition	Vascular relaxation	<10
	H-8	$-(CH_2)_2NHCH_3$	A-PK inhibition	Sperm motility \downarrow	<50
	H-89	$-(CH_2)_2NHCH_2$	A-PK inhibition	Neurite growth \downarrow	<30
	H-7	R2-*2	PK-C inhibition	5HT release 1	<50
	6-H	-(CH ₂),NH,	PK-C inhibition	PK-C purification	<50
	KN-62	· · · · · · · · · · · · · · · · · · ·	CaM KII inhibition	GABA release 👃	<30
	CKI-7	$-(CH_2)_2NH_2$	Casein kinase inhibition	~.	<30
	HA1077	R2-*1	Rho-K inhibition	Vascular relaxation	<10
Others	Staurosporin		Multi kinase inhibition	Ornithine decarboxylase induction	<1.0
	K252a		Multi kinase inhibition	 5HT release ↓	<10
	Sphingolipids		PK-C inhibition	~.	<100
	R1-NHSO ₂	R2-NHSO,	*1	*	
			- H ² C		
		or	HN N- HN N-		

 Table 1
 Protein kinase inhibitors: sulfonamide derivatives and others

shown in Table 1, differing sensitivity toward protein kinases may be mainly dependent on the substituent structure. From the standpoint of protein kinases, our previous study (Hagiwara et al. 1987) suggested that the specificity of the PKA-specific inhibitor, H-8 (isoquinolinesulfonamide), for PKA was related closely to the β -subsite of ATP-binding site of PKA.

2 Rho-Kinase Inhibitor and Its Pharmacological Properties

2.1 Relationship Between Rho-Kinase Inhibition and the Modulation of Physiological Function

Among many isoquinolinesulfonamide derivatives, we selected HA1077 as a therapeutic agent for the treatment of cerebral vascular spasm (Takayasu et al. 1986). This compound (HA1077) was shown to be an excellent therapeutic agent and has already been utilized in 100,000 patients in Japan who suffered from cerebral vascular spasm (Shibuya et al. 1992). The clinical effects of HA1077 are described in another section.

HA1077 was shown to have a wide spectrum of action against various vasoconstriction states including vasospasm. The compound relaxed both the calcium ionophore (A23187)-induced and the receptor agonist-induced contractions of rabbit aorta to a similar extent; the ED_{50} (the concentration of the compound producing 50% inhibition of vasoconstriction induced by various agonists) values were micromolar in potency (Asano et al. 1987). Therefore, to better understand the mechanisms involved, we determined the inhibitory potential on various kinds of enzymes relevant to the contraction mechanism of smooth muscle (Table 2). Like other isoquinolinesulfonamide derivatives, HA1077 inhibited the activity of various protein kinases

\frown	Protein	$K_{\rm i}(\mu{\rm M})$			
HN	kinases	H7	HA1077	H1152	Y-276322
N SO ² CH ³	Rho-kinase	0.457	0.33	0.0016	0.14
CH ₃	PKA	5.7	1.0	0.63	25
	РКС	7.7	9.3	9.27	26
	MLCK	170	55	10.1	>250
N N	PAK ^a	<100	<100	<100	<100
H1152					

 Table 2
 Inhibitory activity of Rho-kinase inhibitors against several kinds of serine/threonine protein kinase

^a p21-Activated kinase.

with various K_i values in ATP-competitive fashion with a range of K_i values. The K_i values for Rho kinase and PKC were comparatively low, 0.35 and 0.90 μ M, respectively. In an isolated arterial strip stimulated with PGF_{2α}, we determined that HA1077 inhibits the phosphorylation of the regulatory myosin light chain (MLC₂₀), in particular the diphosphorylation step was more sensitive than the monophosphorylation reaction (Seto et al. 1991).

HA1077 was an effective inhibitor of arterial contraction and MLC₂₀ diphosphorylation at sub-micromolar concentrations, but the K_i for MLCK was 55 μ M. This fact indicates that the inhibition of MLCK by HA1077 was not likely to be related to its pharmacological action. Subsequently, we and others have demonstrated that MLC diphosphorylation was associated with an inhibition of myosin-phosphatase through the phosphorylation of the myosin binding subunit (MBS 130 kDa) of protein phosphatase 1 by Rho kinase (Kimura et al. 1996; Nagumo et al. 2000). In fact, we demonstrated that HA1077 inhibits PGF_{2a}-induced arterial contraction co-incident with inhibition of MBS-phosphatase (Ito et al. 2003). We believed it was likely that Rho kinase inhibition was likely to be responsible for the mechanism of action of HA1077 and that it could explain its pharmacological activities. Moreover, we discovered that HA1077 also inhibits the phosphorylation of calponin in a porcine coronary artery stimulated with $PGF_{2\alpha}$ (Nagumo et al. 1998). At present, calponin phosphorylation is recognized to be alternatively catalyzed by both PKC and Rho kinase (Kaneko et al. 2000). The regulation of myosin

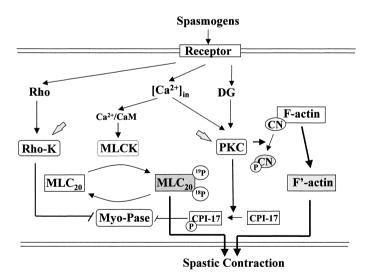


Fig. 7 Site of action of the Rho-kinase inhibitor HA1077 in arterial smooth muscle. *Arrowheads* show the target proteins, Rho-kinase and PKC. *Myo-Pase*, myosin phosphatase 1 with 130 kDa myosin binding subunit; *CN*, calponin; *CPI-17*, 17-kDa inhibitor protein of type 1 phosphatase; *DA*, diacylglycerol

phosphatase activity and calponin phosphorylation, which is dependent on Rho kinase or/and PKC, seems to control the calcium sensitivity of smooth muscle contraction. Taken together, HA1077 may have two sites of action in the mechanism of inhibition of smooth muscle contraction (Fig. 7).

2.2 A Novel Rho-Kinase Inhibitor and Its Perspective

We have made an effort to develop a highly potent and much more specific inhibitor of Rho kinase (Ikenoya et al. 2002). The characteristics of H1152 are summarized in Table 2. In in vitro systems, H1152 potently inhibited Rho kinase activity in a competitive fashion with respect to ATP. As the introduction of two methyl moieties into the HA1077 molecule drastically increases the inhibitory activity against Rho kinase, we analyzed and compared the conformations of H1152 and HA1077 using a computer molecular modeling program. Figure 8 shows that the introduction of the two methyl moieties produces steric hindrance between isoquinoline and homopiperazine moieties, thereby preventing some configurations of H1152 and suggesting that this inhibitor assumes a more rigid configuration than HA1077. In vivo, H1152 specifically inhibited the phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) in human neuronal (NT)-2 cells

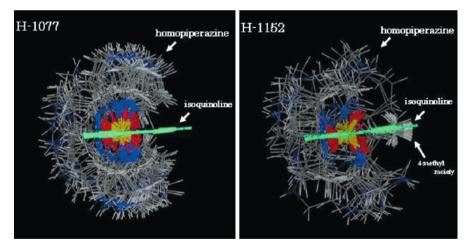


Fig. 8 Comparison of possible conformations of HA1077 and H1152. Systematic conformation analyses and energy minimization, using Chem-X molecular modeling package (version 2000.1; Accerlys K.K. 2–8-4 Shinkawa Chuo-Ku, Tokyo, Japan), assumed stable conformations of HA1077 and H1152. Bonds were rotated in steps of 30°. Energy minimizations were performed employing MM2 force field and default options. The pictures were drawn using MAESTRO software (Schrodinger, Portland OR, USA). (The figure was drawn by Dr. Masami Shiratsuchi and Dr. Hisashi Nakashima, Kowa, Japan, and is cited with permission)

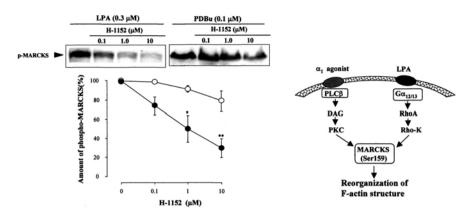


Fig. 9 Inhibition of MARCKS phosphorylation at Ser159 by H1152 in human neuronal (NT)-2 cells stimulated with 0.3 μ M lysophosphatidic acid or 0.1 μ M phorbol dibutyrate (*left panel*). Schematic outline of MARCKS phosphorylation pathways initiated by various stimuli (*right panel*)

stimulated with LPA, but not with phorbol ester (Fig. 9). Previously, we showed that the phosphorylation of MARCKS at Ser159 is catalyzed by Rho kinase as well as PKC (Nagumo et al. 2001). H1152 was used to probe a novel signal transduction pathway for the MARCKS phosphorylation by Rho kinase in LPS-stimulated neuronal cells. MARCKS is assumed to participate in neurotransmitter release through the reorganization or modulation of F-actin structure in neuronal terminals (Trifaro et al. 2002). It is plausible that the F-actin cross-linking activity of MARCKS participates in the dynamics of actin meshwork in a so-called active zone under the presynaptic membranes, and then the reorganization of actin meshwork facilitates the approach of synaptic vesicles to the active zone. Taken together, these data raise the important possibility and hypothesis that a Rho kinase inhibitor may modulate reorganization of the actin-meshwork, suppressing the trafficking of synaptic vesicles and subsequently inhibiting the release of neuro-transmitter.

In neuronal cells and the CNS, Rho kinase is becoming recognized as a potent regulator of neurogenic events, such as the axon elongation and retraction cycle, axon guidance and synaptogenesis, and neurite extension in cultured cells (Kozma et al. 1997; Bito et al. 2000). These concepts seem to be based on the results of molecular and pharmacological experiments using cells expressing a Rho kinase mutant and/or treated with Rho kinase inhibitors. However, little is known about the target molecule of Rho kinase in neuronal cells and CNS.

These is a growing body of evidence that Rho kinase is involved in an increasing number of severe pathologies including angina pectoris (Katsumata et al. 1997), myocardial infarction (Yamamoto et al. 2000), bronchial asthma (Chiba et al. 1999), rheumatoid arthritis, insulin-resistant diabetes mellitus, arteriosclerosis (Fukui et al. 2000), cognitive dysfunction (unpublished data), and astrocyte activation (Abe and Misawa 2003). Thus, with the discovery of novel substrates of Rho kinase, highly potent and specific forms of Rho kinase may be recognized not only in normal but also in pathological conditions. Rho kinase inhibitors may be very important as medicines for such diseases as well as diagnostic probes for Rho kinase-involved physiology (Moolenaar et al. 1997).

3 Clinical Application of Isoquinolinesulfonamide to Human Cerebral Vascular Diseases

Cerebral vasospasm is the leading cause of poor postoperative outcome of patients with subarachnoid hemorrhage due to ruptured aneurysms. Constriction of the arterial lumen begins about 4 days after the hemorrhage. Patients begin to show symptoms and signs of spasm at about the seventh day which last for 2-3 weeks. However, the spastic arteries begin to dilate by themselves after the fourth week if the patient can tolerate the ischemic insult. The primary cause of spasm is the degradation products of red blood cells in subarachnoid clot. However, further details of the mechanism have vet to be clarified. Both constriction of smooth muscle cells and inflammation seem to be involved. After the patient is secured from further bleeding by a successful neck clipping or intravascular treatment by GDC (Guglielmi detachable coil), 30% of the patients suffer from vasospasm in various forms, resulting in neurological deficits that range from mild cognitive dysfunction, sensorimotor paralysis, and aphasia, to death. Although, nimodipine, a calcium entry blocker, is widely used in the Western world, its effect has not always been satisfactory. HA1077 has been used in about 12,000 patients each year since it was approved in Japan in 1995. A recent comparative study in China between HA1077 and nimodipine on patients with subarachnoid hemorrhage (SAH) showed that HA1077 is equal to or better than nimodipine (unpublished data).

In this chapter, clinical effects as well as pharmacological analysis of HA1077 using model animals with experimental vasospasm or cerebral infarction will be further described.

3.1

Demonstration of the Therapeutic Effect of HA1077 on Cerebral Vasospasm and Infarction Using Model Animals

3.1.1 Effect on Subarachnoid Hemorrhage

Mongrel dogs of either sex weighing 7.5–13 kg or male beagle dogs weighing 7–10 kg were used as a two hemorrhage canine model of SAH. These model dogs reliably produce a chronic and intractable cerebral vasospasm similar to that seen in humans. A significant vasospasm was observed angiographically on day 7 after SAH in every dog. The diameter of the basilar artery diminished to 50%–60% of the control value obtained before SAH (on day 1). Chronic cerebral vasospasm in this model was not reversible by intravenous high doses of aminophylline, nifedipine, nimodipine, or papaverine (Varsos et al. 1983).

The intravenous administration of 0.5–10 mg/kg/30 min HA1077 caused a dilation of the basilar artery dose-dependently on day 7. HA1077 lowered the mean arterial blood pressure only at high doses of 3 and 10 mg/kg (Takayasu et al. 1986).

Prophylactic administration of HA1077 infused intravenously for 30 min twice daily from day 1 after SAH significantly prevented the development of chronic cerebral vasospasm, as demonstrated by angiography (Fig. 10). SAH produced a significant decrease in regional cerebral blood flow (rCBF), and blood velocity, with no remarkable change in blood volume. These results were supported by previous clinical investigations showing similar observations in patients with SAH (Grubb et al. 1977; Yamakami et al. 1983). This phenomenon is probably explained by the pathophysiology whereby the large, radiographically visible vessels contract without contraction of the arteriolar resistance vessels. A bolus intravenous injection of HA1077 dose-dependently increased the rCBF and blood velocity, without significantly changing the blood volume on day 7 after SAH.

It has been known that SAH induces marked pathological changes in cerebral arteries. In the light micrographs and transmission electron micrographs of the basilar arteries, severe constriction of the artery plus folding and corrugation of the elastic lamina were apparent. The endothelial cells were compressed between tight folds of the elastic lamina during a period of prolonged contraction, and these physiological mechanisms may provoke endothelial damage. Scanning electron micrographs of the endothelial surface of the basilar artery demonstrated endothelial disruption such as platelet aggregation, adhesion of red blood cells and leukocytes to the endothelial surface, and endothelial desquamation. Substances released from the platelets and leukocytes adhering to the luminal surface accelerate the constriction of spastic artery. The blood velocity may increase in a narrow segment

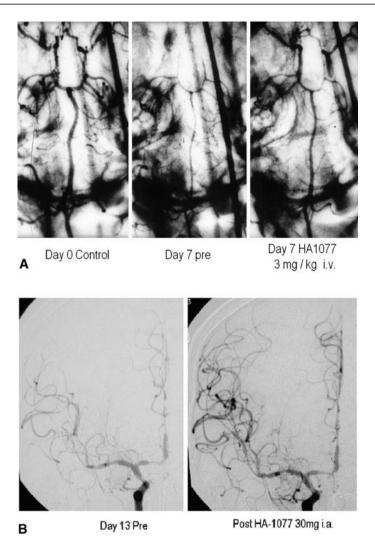


Fig. 10 A Effect of intravenous HA1077 on canine vasospasm. *Left*, control angiogram on day 0 before the subarachnoid blood injection. *Center*, day 7 before HA1077, shows a marked narrowing of the basilar artery. *Right*, 30 min after intravenous administration of 3 mg/kg HA1077, shows marked dilatation of the basilar artery. **B** Effect of intra-arterial administration of HA1077 in a patient with a ruptured fusiform aneurysm in the basilar artery. *Left*, right carotid angiogram on day 13 shows multiple segmental narrowing in the carotid, anterior, and middle cerebral arteries. *Right*, marked dilatation of the spastic arteries is seen after intra-arterial administration of 30 mg of HA1077. Patient's consciousness level improved after HA1077

of spastic vessels. This higher velocity of blood flow increases shear stress and damages the endothelial surface. However, pathological damage of cerebral arteries in HA1077-treated dogs was absent or minimal in contrast with the control dogs (Satoh et al. 1999a).

Activated neutrophils may play a major role in the pathogenesis of cerebral arterial vasospasm or cerebral injury. Such processes cause neutrophils to degranulate, aggregate, adhere to the endothelium, infiltrate, and release highly reactive compounds that promote inflammatory local responses. Oxygen radicals released from neutrophils are considered to be toxic to endothelial cells and to cause endothelial damage. Additionally, it is considered that free radicals from neutrophils induce cellular injury with inactivation of nitric oxide, a known vasodilator (Kajita et al 1994). We reported that inactivation of nitric oxide by superoxide anions contributes to the development of vasospasm (Suzuki et al. 1992). A later report (Arai et al. 1993) demonstrated that HA1077 can prevent the production of superoxide anions in neutrophils as well as the migration of neutrophils.

3.1.2 Effect on Cerebral Ischemia

The neuroprotective properties of HA1077 were evaluated in two animal models of cerebral ischemia: transient bilateral carotid artery occlusion in Mongolian gerbils and cerebromicroembolization in rats (Satoh et al. 1996). The common carotid arteries in male gerbils were clamped with microclips for 5 min. The brains were removed 7 days later and coronal sections through the hippocampus were prepared and stained with hematoxylin and eosin. The degree of neuronal damage was assessed in the CA1 region of the hippocampus by quantifying the number of pyramidal cell bodies per millimeter. The average of the neuronal cell density of the normal gerbil was 211 ± 5 cells/mm, and the average in the ischemic control group of gerbils was 17.8 ± 2.1 cells/mm. HA1077 significantly diminished the loss of CA1 neurons in hippocampus with the average of neuronal cell density of 101.0 ± 22.0 cells/mm. Nimodipine, a calcium entry blocker and ozagrel, a thromboxane A₂ synthase inhibitor, did not significantly protect against the ischemia-induced neuronal loss.

Multiple infarctions were produced in the cerebral hemispheres of rats by the injection of the 50- μ M microspheres into the left internal carotid artery, and neurological impairment was evaluated 24 h later. The assessment of neurological function following cerebral microembolization is a subjective test and would only give an indication as to alterations in function, but all rats in the control group showed typical symptoms of stroke such as truncal curvature, circling behavior, and rolling behavior. Although distribution of the infarction was unpredictable, the structures most frequently involved were the hippocampus, thalamus, and cortex, and the total size of infarctions was comparatively constant.

Neurological function and size of infarction was significantly improved in the HA1077-treated animals. HA1077 also suppressed the increased water content in ischemic brain tissues. Administration of HA1077 significantly improved the ischemia-induced impairment of neurological function, as well as impairment in the morphology.

It has been reported that the intravenous infusion of HA1077 increased rCBF and glucose utilization at various sites, including thalamus, cortex, and hippocampus (Sako et al. 1991; Tsuchiya et al. 1993). These sites were consistent with the infarct areas. The results suggest that HA1077 protects the brain from ischemic infarction by improving hemodynamic and metabolic function, at least in part.

Neutrophil accumulation in cerebral ischemic infarcts was observed in the ipsilateral hemisphere of ischemic brains, while there were few neutrophils in the normal brain area. Accumulation of leukocytes is known to be greater in the region of more severe ischemia and the central zone of the ischemia in acute embolic stroke in humans. Neutrophil infiltration into the ischemic area of the brain may be involved in the pathogenesis of ischemic injury and neurological impairment. As HA1077 can prevent the production of superoxide anions in neutrophils as well as the migration of neutrophils, this may be an important part of mechanism in neuroprotective effects of HA1077 (Satoh et al. 1999b). HA1077 had no significant effect on the magnitude of body temperature. This suggests that neuroprotective effect of HA1077 is not due to hypothermia.

3.2 Clinical Application of HA1077 to 100,000 Patients

3.2.1 Anti-vasospasmic Effect

HA1077 is unique in dilating spastic arteries not only by local (intra-arterial) application but also by systemic (intravenous, i.v.) administration with doses not causing significant hypotension (Takayasu et al. 1986). Usual calcium entry blockers such as nicardipine or diltiazem result in poor vasodilating effects even in doses that show significant hypotension. In order to select an appropriate dosage for use in a double-blind trial, various doses of HA1077 (20–180 mg/day) were given to separate groups of patients. Each dose of the drug was given in 100 ml of saline, infused for 30 min, two or three times a day for 14 days after clipping the aneurysm. Severe angiographic spasm decreased with increasing doses of HA1077. No severe vasospasm was seen in a group receiving 40–60 mg, three times a day. However, patients on high doses of HA1077 occasionally suffered mild hypotension,

	Placebo	HA1077	p Value
1. Angiographic vasospasm (mod. or sev.)	61%	38%	<i>p</i> <0.01
2. Symptomatic vasospasm	50%	35%	p<0.05
3. Low-density area on CT scan (mod. or sev.)	38%	16%	p<0.01
4. Poor ADL (<vasospasm)< td=""><td>26%</td><td>12%</td><td>p<0.05</td></vasospasm)<>	26%	12%	p<0.05
All causes	40%	34%	n.s.
5. Adverse reactions	6%	4%	n.s.

Table 3	Summary:	double-blind	trial of fasu	dil (HA1077)
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Mod., moderate; n.s., not significantly different; sev., severe.

especially dehydrated, small or older patients. No other side effects have been reported (Shibuya et al. 1990). Thus, three doses of 30 mg per day for 14 days was chosen for the double-blind trial. A placebo-controlled prospective double-blind trial was performed in 280 patients whose aneurysm was clipped within 3 days after the hemorrhage (Shibuya et al. 1992). The results of the double-blind trial (Table 3) showed that HA1077 significantly decreased symptomatic spasm from 50% to 35% (p<0.05), angiographic spasm from 61% to 38% (p<0.01, Fig. 11), and cerebral infarction which was seen as a low density area (LDA) on computed tomography (CT) from 38% to 16% (p<0.01). Poor outcome or ADL (active daily life) due to vasospasm was decreased from 26% to 12% (p<0.05). HA1077 is the only drug that showed significant effects by angiography and CT scan. Similar effects of

Fasudil DBT: Angiographic vasospasm

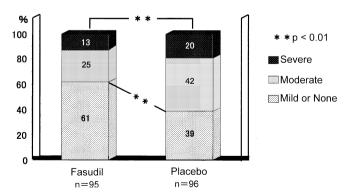


Fig. 11 Incidence of angiographically detected vasospasm. The patients in the fasudil (HA1077) group were administrated three times 30 mg HA1077 each day for 14 days, and the patients in placebo were given physiological saline. Values are percentages of the total within each group. Significant differences for moderate and severe spasm were found: p=0.0023

HA1077 have been shown in patients after it was commercialized. The currently approved dosage of HA1077 (30 mg, i.v., three times a day, for 14 days), HA1077 can ameliorate about half of the damage caused by vaso-spasm. This is an encouraging result and further improvement can be expected by increasing the dosage of HA1077. However, careful titration of the dosage of HA1077 must be undertaken to guard against hypotension. A close watch for fluid balance is also important, since HA1077 sometimes causes dehydration and hemoconcentration by increasing renal blood flow and urine volume, which may cause a further deterioration of ischemic injury in patients with vasospasm.

3.2.2 Intra-arterial Use of HA1077

Even when receiving intravenous treatment with HA1077, there are some patients who develop signs of vasospasm, usually at about 7–10 days after the hemorrhage. Intra-arterial use of HA1077 is effective in such cases. Thirty to 60 mg of HA1077 is given in divided doses through the arterial catheter during DSA (digital subtraction angiography). Intra-arterial HA1077 can dilate most of the spastic arteries. However, its clinical usefulness is limited to about 50% of the patients, because its effect may be only short acting or infarction had already been completed (Tachibana et al. 1999). The vasodilating effect of HA1077 is similar to that of papaverine, but HA1077 is superior to papaverine with fewer side effects. The only side effect reported with intra-arterial administration of HA1077 is seizure, especially when a large dose (more than 100 mg) is used in a short period. Such seizures can be treatable with diazepam.

3.2.3

Effect of HA1077 on Cerebral Infarction

In the above-mentioned double-blind trial of HA1077 in patients with SAH, the brain-protective effect seen by a decrease in cerebral infarction on CT scans was most remarkable (Table 3). In experiments in rats, the effects of HA1077 on cerebral infarction were shown in the carotid occlusion model (Tsuchiya et al. 1993; Satoh et al. 1999a). The effect of HA1077 on cerebral ischemia is twofold. One positive effect is an increase in collateral circulation in penumbra through dilation of the cerebral arteries, a second positive effect is an anti-inflammatory action. HA1077 has been shown by HMPAO (Tc-99m hexamethyl propyleneamine oxime) to increase rCBF in patients after SAH. Interestingly, it increased rCBF in the region of cerebral vasospasm without changing blood flow in normal regions. In other words, it increased rCBF more specifically in spastic regions, thus avoiding development of a so-called steal phenomenon. This is unique to HA1077. It is usual for calci-

um entry blockers to dilate normal arteries more than spastic arteries causing systemic hypotension, which is generally not desirable.

HA1077 had been shown to inhibit migration of white blood cells to the ischemic brain in rats (Satoh et al. 1999a,b). In addition, in in vitro system, HA1077 inhibits production of free radicals by NADPH oxidase in WBC through the inhibition of PKC (Arai et al. 1993). Decrease of free radical production by inhibition of PKC seems to be an important part of the effectiveness of HA1077. As HA1077 inhibits the phosphorylation of calponin in the intact artery, the effects of HA1077 on both cerebral vasospasm and infarction can be attributed to both vasodilatation and anti-inflammation. In a recent clinical study, a double-blind trial of HA1077 in patients with acute cerebral infarction due to thrombosis showed that HA1077 significantly decreased motor weakness and improved ADL (unpublished data). Effectiveness of HA1077 has experimentally been suggested in many other diseases where Rho kinase is believed to be involved, including angina pectoris (Katsumata et al. 1997), bronchial asthma (Chiba et al. 1999), rheumatoid arthritis, insulin-resistant diabetes mellitus, and arteriosclerosis (unpublished data).

In conclusion, HA1077 was found to be a novel compound inhibiting Rho kinase. This compound has been used clinically, especially for the therapy of cerebral vasospasm or cerebral infarction. Studies of model systems in animals with vasospasm or cerebral infarction have provided evidence to advance the study of HA1077 into the therapy of human vasospasm or cerebral infarction. Clinical trials of nearly 100,000 patients treated with HA1077 in Japan since 1995 have demonstrated that the Rho kinase inhibitor, HA1077 is quite a useful agent for the treatment with vasospasm.

Rho kinase has recently been considered one of the key enzymes involved in the development of vasospasm. The inhibition of Rho kinase by HA1077 has opened the door to an enhanced understanding of mechanisms involved not only in cerebral vasospasm and infarction but also many other diseases. The studies also have the potential to open an entirely new generation of therapeutic opportunities and important tools to study disease pathways and mechanisms.

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Discovery and Development of Iressa: The First in a New Class of Drugs Targeted at the Epidermal Growth Factor Receptor Tyrosine Kinase

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Abstract The epidermal growth factor receptor (EGFR) is a promising target for anti-cancer therapy because of its role in tumour growth, metastasis and angiogenesis, and tumour resistance to chemotherapy and radiotherapy. This chapter describes a low-molecular-weight EGFR tyrosine kinase inhibitor (EGFR-TKI), Iressa (gefitinib, ZD1839). Iressa is a potent EGFR-TKI which blocks EGF-stimulated EGFR autophosphorylation in tumour cells and selectively inhibits EGF-stimulated tumour cell growth. In studies with mice bearing a range of human tumour-derived xenografts, Iressa given orally, once daily, inhibited tumour growth in a dose-dependent manner. Long-term (>3 months) Iressa treatment of xenograft-bearing mice was well tolerated, and Iressa completely inhibited the growth of xenografts derived from A431 cells which highly express EGFR, and induced regression of advanced A431-derived tumours. No Iressa-resistant tumours appeared during treatment, but some tumours regrew following drug withdrawal. The level of expression of EGFR did not determine xenograft tumour sensitivity to Iressa. These preclinical studies indicated the potential utility of Iressa in the treatment of a wide range of human tumours, and established that continuous once-daily oral dosing might be a suitable therapeutic regimen. The first clinical studies with Iressa confirmed that an oral, once-daily, regimen is generally well tolerated and shows activity in cancer patients. Phase II clinical trials, in patients with advanced non-small-cell lung cancer (NSCLC) who had been previously treated with chemotherapy, demonstrated that Iressa at a daily dose of 250 or 500 mg has a favourable safety profile and has activity. The lower (250 mg/day) dose was better tolerated and as effective as the 500 mg/day dose. In these phase II studies, the level of expression of EGFR did not predict response to Iressa treatment. In phase III clinical trials, addition of Iressa to standard two-drug chemotherapy regimens, in previously untreated NSCLC patients, failed to improve disease-free or overall survival. Studies are now underway to investigate how Iressa can be used as an alternative to or in sequence with chemotherapy in NSCLC, and to explore its efficacy in other tumours including head and neck, breast and colorectal.

Keywords Iressa · Gefitinib · ZD1839 · Epidermal growth factor receptor · EGFR tyrosine kinase inhibitor · NSCLC

Iressa is a trademark of the AstraZeneca group of companies.

1 Rationale

The application of molecular genetic techniques to cancer biology has elucidated multiple, interacting biochemical pathways involved in the malignant transformation and unrestricted growth of epithelial cells from which the majority of human solid tumours originate (Hunter 1997; Blume-Jensen and Hunter 2001). The first complete description of a biochemical pathway originating at the cell surface, propagating signals from the tumour cell environment and leading to changes in gene transcription and tumour cell proliferation, began with the recognition that the epidermal growth factor receptor (EGFR) is the normal cellular homologue (proto-oncogene) of the viral oncogene v-erbB, the oncogenic protein of the avian erythroblastosis virus (Downward et al. 1984). This description culminated in the recognition that the ras-raf-mitogen-activated protein kinase (MAPK) pathway transmits signals activated by binding of EGF or transforming growth factor alpha (TGF- α) to the EGFR (Schlessinger 1993). At the same time, the first reports that squamous cell carcinomas express high levels of EGFR at the cell surface (Gusterson et al. 1984), and that the presence of EGFR indicates a poor prognosis in breast cancer patients (Sainsbury et al. 1985), were published. Subsequently, it became clear that high levels of EGFR expression are a common manifestation of the malignant phenotype in many solid human tumours including those of the lung (Gullick 1991; Salomon et al. 1995; Nicholson et al. 2001).

The EGF receptor (also designated ErbB1/HER1) is the prototype of a large family of cell-surface receptor tyrosine kinases (RTKs) that possess intrinsic tyrosine kinase activity and transmit growth factors signals across the cell membrane. The ErbB family of RTKs has four members (EGFR/ ErbB1/HER1, ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4), and at least ten ligands which include EGF, transforming growth factor (TGF)- α and the heregulins. These ligands preferentially induce different ErbB homo- and hetero-dimers conferring a high degree of signalling diversity (Yarden and Ulrich 1988; Olayioye et al. 2000; Yarden and Sliwkowski 2001). RTKs share a common structure encompassing an extracellular ligand-binding domain,

a short transmembrane domain and an intracellular domain which has tyrosine kinase (TK) activity. Binding of EGF or TGF- α to EGFR stimulates receptor homo- or hetero-dimerisation, intramolecular autophosphorylation of specific tyrosine residues of EGFR (or other ErbBs in heterodimers) providing 'docking sites' for the assembly of signal transduction complexes, and direct phosphorylation of specific tyrosine residues in substrate proteins (Yarden and Ulrich 1988). Delineation of the molecular mode of action of the EGFR, together with the demonstration that mutation or overexpression of ErbB2, as well as EGFR, causes cellular transformation and is associated with poor prognosis in breast cancer (Slamon et al. 1987), provided a convincing case that these receptors represent a potential target for novel anti-tumour therapies. Proof of principle for this idea was first provided by the successful application of trastuzumab (Herceptin), a monoclonal anti-ErbB2 antibody, in the treatment of breast cancer patients whose tumours overexpress ErbB2 (Cobleigh et al. 1999). An alternative therapeutic approach is directed at inhibition of the EGFR tyrosine kinase (EGFR-TK) activity with small molecular weight enzyme inhibitors (Boschelli 1999; Woodburn 1999; Ciardiello and Tortora 2001; Grünwald and Hidalgo 2003).

Because the cytoplasmic tyrosine kinase domains of the receptor protein tyrosine kinase 'super family' are highly homologous, particularly within sub-families like the ErbB/HER family, an important issue at the beginning of the drug discovery program which led to Iressa, was whether or not it would be possible to find inhibitors selective for EGFR. An encouraging precedent for a synthetic chemistry-based approach to EGFR-TK inhibition was provided by the work of Yaish et al. (1988), who described small molecule inhibitors of EGFR-TK activity which did not inhibit the TK activity of the insulin receptor. Since RTKs are expressed in normal tissues as well as in tumours (Yano et al. 2003), selectivity for EGFR was deemed an important attribute for a drug candidate molecule in order to reduce as far as possible the likely undesirable sequelae of generic inhibition of receptor tyrosine kinase activity. The potential issue of toxicity associated with EGFR blockade in normal tissues was recognised; however, it was felt that the high level of activity of EGFR signalling in tumours implied by overexpression of the EGFR, compared with normal tissues, could provide an opportunity to define an acceptable therapeutic ratio between efficacy and toxicity. By analogy with previous experience of oestrogen receptor-targeted treatment of breast cancer with tamoxifen (Nolvadex) and fulvestrant (Faslodex), the aim was to develop a well-tolerated cytostatic agent suitable for long-term use in cancer patients. A drug discovery program was initiated in 1990 at ICI Pharmaceuticals (the predecessor company of Zeneca Pharmaceuticals, in turn a predecessor of AstraZeneca Pharmaceuticals), using a biochemical screen to test compounds representative of the chemical diversity in the company compound inventory, in a substrate tyrosine phosphorylation assay, with EGFR-TK prepared from a human tumour cell line (A431) that highly expresses EGFR. Lead identification was enhanced by the application of structure-based searching (Ward et al. 1994). The discovery of potent selective EGFR-TK inhibitors of the anilinoquinazoline class (Barker and Davies 1992; Fry et al. 1994; Wakeling et al. 1996) was the first step towards selecting a candidate drug molecule, ZD1839, with which to test the efficacy-versus-toxicity question (Barker et al. 2001). This chapter describes the properties of Iressa, (gefitinib, ZD1839), an orally active EGFR-TK inhibitor, and clinical experience to date in cancer therapy with Iressa.

2 Pharmacology

2.1 Enzyme Inhibition

Chemical compounds were tested as potential inhibitors of EGFR-TK activity in a substrate phosphorylation assay with a synthetic peptide substrate and a plasma membrane preparation derived from human A431 cells, which overexpress the EGFR. This assay first identified the anilinoquinazoline class of EGFR-TK inhibitors (Ward et al. 1994; Wakeling et al. 1996), and subsequently the candidate drug EGFR-TK inhibitor, ZD1839 (Gibson et al. 1997; Barker et al. 2001; Wakeling et al. 2002), designated Iressa. Iressa potency and kinase selectivity was demonstrated by comparing its activity against EGFR-TK with that against several other tyrosine and serine-threonine kinases (Wakeling et al. 2002). Enzyme kinetic methods described by Ward et al. (1994) showed that Iressa is competitive with ATP (K_i =2.1±0.2 nM) and non-competitive with substrate (K_i =15.0±1.0 nM). Thus, Iressa inhibits EGFR-TK activity by competing directly with ATP at the enzyme active site. EGF binding to EGFR stabilises a kinase active dimeric form of EGFR and stimulates intermolecular autophosphorylation of receptor homo- or hetero-(erbB)-dimers. Iressa inhibits EGF-stimulated EGFR autophosphorylation in several human tumour cell lines cells (Wakeling et al. 2002). Iressa blocked EGF-stimulated EGFR autophosphorylation in a dose-dependent and complete manner; complete inhibition was achieved at 0.16 μ M in Du145 (prostate) and A549 (lung) cells, and at 0.8 μ M Iressa in KB (oral squamous) and HT29 (colon) cells. Drug wash-out studies showed that this inhibition was sustained for at least 24 h following a 2-h drug treatment.

Investigation of TK inhibitory selectivity in cell lines expressing EGFR or ErbB2 showed that Iressa, at concentrations up to 5 μ M, does not directly inhibit ErbB2 tyrosine phosphorylation (Christensen et al. 2001; Moulder et al. 2001; Normanno et al. 2002).

2.2 Inhibition of Tumour Cell Growth In Vitro

An important aspect of our drug discovery strategy required a test which would distinguish between specific EGFR-TKI-mediated effects on cell growth and non-target-specific cell growth inhibition or cytotoxicity. For this purpose we selected the human KB cell line derived from a vulval squamous tumour (Wakeling et al. 1997). KB cells grow well in basal conditions, that is in growth medium supplemented with 5% charcoal-treated calf serum (to deplete serum growth factors), and redouble their growth rate upon addition of EGF to the growth medium. Iressa inhibited EGF-stimulated growth much more potently than basal growth (IC₅₀ 0.054 μ M compared with 8.8 μ M) demonstrating that Iressa specifically inhibits EGF-stimulated tumour cell growth (Wakeling et al. 2002).

Selectivity of cell growth inhibition by Iressa was also exemplified in studies with normal tissue-derived human cells (HUVEC, human umbilical vein endothelial cells) which are stimulated to grow by several different growth factors. Iressa inhibited EGF-stimulated HUVEC cell growth with a potency similar to that recorded for EGF-stimulated KB cells (<0.1 μ M) whereas at least tenfold greater concentrations were needed to significantly affect FGF- or VEGF-stimulated HUVEC growth (Wakeling et al. 2002).

Numerous investigators have shown that Iressa inhibits the proliferation of many different tumour cell lines; this growth inhibition is accompanied by inhibition of EGFR phosphorylation and of downstream signalling through the ERK MAP kinase(s) and PKB/Akt pathways, but tumour cell sensitivity to Iressa was not determined by the level of EGFR expression (Ciardiello et al. 2000; Albanell et al. 2001; Anderson et al. 2001; Moasser et al. 2001; Barnes et al. 2003; Vicentini et al. 2003). Although Iressa does not directly inhibit ErbB2 phosphorylation, it effectively inhibits the growth of ErbB2-overexpressing tumour cells, and inhibitory efficacy did not depend on the relative levels of expression of ErbB2 and EGFR (Anderson et al. 2001; Moasser et al. 2001; Moulder et al. 2001). Inhibition of ErbB2 signalling by Iressa may be mediated by induction of inactive ligand-heteromeric receptor complexes containing ErbB2 and ErbB3 (Lichtner et al. 2001; Anido et al. 2003). Thus, Iressa may inhibit heregulin-mediated tumour cell growth as well as that mediated by EGFR ligands. As well as inhibiting tumour cell proliferation, Iressa treatment increases apoptosis (Ciardiello et al. 2000; Gilmore et al. 2002; Normanno et al. 2002), reduces motility and invasiveness (Fujimura et al. 2002; Barnes et al. 2003) and decreases angiogenesis in some tumour cells (Ciardiello et al. 2001; Hirata et al. 2002). Iressa is a particularly effective inhibitor of the growth of anti-oestrogen-resistant breast cancer cells (McLelland et al. 2001; Knowlden et al. 2003), of taxaneresistant, hormone-independent breast cancer cells (Ciardiello et al. 2002) and enhances the anti-tumour activity of cytotoxic drugs (Ciardiello et al.

2001, 2002; Magne et al. 2002) and of radiation treatment (Bianco el 2002; Huang et al. 2002; Williams et al. 2002) in vitro.

2.3 In Vivo Anti-tumour Activity

Testing of drug candidate TK inhibitors for in vivo anti-tumour activity was carried out with KB cell-derived human squamous tumour xenografts grown in nude mice. Structural chemistry necessary for in vivo anti-tumour activity, and the importance of sustaining concentrations of drug in the blood sufficient to inhibit EGFR-TK activity throughout each 24-h period following once-daily, oral dosing, were defined using this model. Iressa was chosen as a drug development candidate because it achieves high and sustained blood levels, in vivo, over a 24-h period (Barker et al. 2001). The potential spectrum of anti-tumour activity for Iressa in patients was assessed in xenografts of major solid human tumours representing a wide range of tissues of origin (Wakeling et al. 2002). The anti-tumour efficacy of Iressa varied widely between tumours. Dose-dependent growth inhibition was noted for A431 (human vulval squamous), A549 (lung), Du145 (prostate), HCT15, HT29 and LoVo (colon), and KB (oral squamous) tumours (dosing regimen 3-200 mg/kg, po, once daily). Maximum anti-tumour efficacy ranged from tumour regression of A431, through tumour stasis in A549 and Du145 tumours, to no effect on P246, (broncho-epithelial), MKN45, (gastric) and AR42 J, (pancreatic) tumours. No correlation was apparent between xenograft response to Iressa and expression levels of EGFR in the cognate tumour cell lines.

In long-term treatment studies, Iressa provided complete control of tumour growth; when drug treatment was withdrawn after 4 months, most but not all tumours resumed growth, demonstrating that Iressa has a cytostatic effect. When drug treatment was delayed until tumours were well-established, Iressa caused complete regression of all A431 tumours. Drug withdrawal again allowed tumour regrowth. No evidence for the development of drug resistance emerged during these studies with A431 tumours, since no tumour regrew during Iressa treatment (Wakeling et al. 2002). To assess the pharmacodynamic action of Iressa treatment, A431 tumours were excised 6 h after the last of four daily doses of 0, 12.5, 50 and 200 mg/kg Iressa, or at 2, 4, 6, 24, 30 and 36 h after a single 50 mg/kg dose. Total RNA was extracted from the tumours to quantify c-fos mRNA by RT-PCR. C-fos expression was inhibited in a dose-dependent manner; at the anti-tumour ED₅₀ dose (50 mg/kg), c-fos mRNA was reduced by 94% and was completely blocked (>99%) at the 200-mg/kg dose. In mice treated with a single 50-mg/kg po dose of Iressa, c-fos expression reached a nadir (5% versus control) at 6 h and did not fully recover until 36 h later. These studies were consistent with

a cytostatic effect on tumour cell growth, and suggested that continuous drug treatment is required to maintain control of tumour growth.

Xenograft studies have demonstrated Iressa's potential clinical efficacy alone (Anderson et al. 2001; Moasser et al. 2001; Fujimara et al. 2002; Sewell et al. 2002; Matsuo et al. 2003), in combination with single agent chemotherapy (Ciardiello et al. 2000; Sirotnak et al. 2000), radiotherapy (Bianco el 2002; Huang et al. 2002; Williams et al. 2002) or hormone therapy (Sirotnak et al. 2002; Schiff et al. 2004), and in combination with other targeted treatments (Moulder et al. 2001;Tortora et al. 2003). Animal model studies have also demonstrated that Iressa may have utility in early disease, for example, in the adjuvant or chemopreventative treatment of breast cancer. Iressa inhibited the proliferation of xenografts of normal breast and of ductal carcinoma in situ (Chan et al. 2001, 2002), and suppressed the development of mammary tumours in mouse mammary tumour virus (MMTV)-c-erbB2 transgenic mice (Lu et al. 2003). Since no correlation has been established between response and expression of EGFR in any of these studies, it is clear that receptor expression does not indicate the degree to which any individual tumour is dependent for growth on the EGFR signalling pathway. Biomarkers that might predict drug response or resistance have yet to be defined. Some studies have implicated persistent activation of the AKT/PKB pathway in resistance to EGFR-TK inhibition. For example, loss of PTEN (Bianco et al. 2003), high AKT activity (Janmaat et al. 2003) and increased insulin-like growth factor (IGF)-1R signalling (Chakravarti et al. 2002) all confer decreased sensitivity to anti-EGFR treatments. Comparison of gene expression profiles in Iressa-sensitive and -resistant tumour cell lines or xenografts could provide markers of activity for evaluation in biopsies from drug treated patients (Zembutsu et al. 2003).

3 Clinical Studies

3.1 Phase I Trials

Early investigations of the pharmacokinetics and oral bioavailability of Iressa were carried out in healthy male volunteers (Swaisland et al. 2001). These studies demonstrated that peak plasma drug concentration occurred 3–7 h after administration, the concentration-time curve was dose-proportional from 10- to 100-mg doses, and the terminal elimination half-life was 28 h. These studies indicated the potential for a convenient once-daily oral administration schedule. In preliminary studies to investigate the efficacy and tolerability of Iressa, patients with a variety of solid tumours were treated once daily with oral doses up to 1,000 mg of Iressa (Baselga et al. 2002; Herbst et al. 2002; Ranson et al. 2002; LoRusso et al. 2003; Nakagawa et al. 2003). Iressa was generally well-tolerated; the most common side effects were mild to moderate diarrhoea and rash (grade 1/2), which was reversible. The incidence and severity of side effects increased with increasing dose, but there were none of the severe adverse events involving the haemopoietic, digestive and nervous systems commonly associated with cytotoxic chemotherapy. The maximum tolerated dose (MTD) was greater than or equal to 700 mg/day. Anti-tumour activity was observed in a number of tumour types, notably non-small-cell lung cancer (NSCLC; 10/100 patients with partial response), at doses exceeding or equal to 150 mg/day. Consistent with these clinical observations, biomarker studies in skin samples taken from Iressa-treated patients showed that Iressa inhibited EGFR action to a similar extent at all doses meeting or exceeding 150 mg/day (Albanell et al. 2002). Iressa inhibited EGFR and MAPK phosphorylation, reduced keratinocyte proliferation index and increased apoptosis and expression of the CDK inhibitor p27^{KIP1} and of STAT3. Dose selection for further study was intended to identify a dose for optimum anti-tumour activity, combining maximum efficacy with minimum adverse events, and avoiding the classical MTD approach generally applied to the study of novel cancer chemotherapeutics. Thus, two doses below the MTD, 250 mg/day and 500 mg/day, were chosen for evaluation in phase II trials. Responses with minimum toxicity had been seen at the 250-mg/day dose, and the 500-mg/day dose was the highest welltolerated dose during prolonged treatment.

3.2 Phase II Trials

Following-up the encouraging responses seen in NSCLC patients in the phase I studies, two phase II clinical trials of Iressa monotherapy were conducted in patients with locally advanced or metastatic NSCLC who had previously been treated with platinum-based chemotherapy (Fukuoka et al. 2003; Kris et al. 2003). Trials IDEAL 1 and 2 (Iressa Dose Evaluation in Advanced Lung Cancer) evaluated two different doses, 250 and 500 mg/day, which were given daily until disease progression or withdrawal due to intolerable toxicity. Patients in IDEAL 1 were recruited from Europe, Japan, Australia and South Africa and had received one or two prior chemotherapy regimens, at least one of which contained platinum. Patients in IDEAL 2 were recruited from the USA and had received at least two prior regimens including platinum and docetaxel given either concurrently or separately. Both doses of Iressa produced similar anti-tumour effects but side effects were fewer and less severe at the lower dose. For the 250-mg/day dose, response rates in IDEAL 1 and 2 were 18.4% and 11.8%, respectively. Since many NSCLC patients have frequent and severe symptoms, the impact of treatment with Iressa on symptoms was investigated. Symptom improvement was recorded in approximately 40% of patients (40.3% in IDEAL 1 and 43.1% in IDEAL 2), and such improvements occurred very rapidly with a median time to improvement of 8 and 10 days after beginning Iressa treatment (Douillard et al. 2002). Symptom improvement correlated with objective tumour response and was associated with increased overall survival; for example, in IDEAL 2, median overall survival in patients with symptom improvement was 13.6 months, compared with 3.7 months for patients without symptom improvement. The most frequent drug-related adverse events in the IDEAL trials were skin rash (47% and 43% in IDEAL 1 and 2, respectively) and diarrhoea (40% and 48%), which were generally mild in nature (grade 1/2).

Determination of tumour EGFR status was not an entry criterion for the IDEAL trials, but tumour biopsies taken during these trials have been analysed for EGFR expression. An immunohistochemical (IHC) assay estimated the intensity of EGFR membrane staining intensity using a four-point scale (0, 1+, 2+, 3+ for no, weak, moderate and strong staining, respectively) (Janas et al. 2003). The correlation of membrane staining intensity with the probability of objective tumour response or symptom improvement was investigated. The mean proportion of cells staining 2+ or 3+ in tumours from patients who responded was 31.3% compared with 37.5% in those who did not respond. In both trials there were patients who responded but in whom tumour EGFR expression was very low (Bailey et al. 2003). Therefore, this analysis failed to reveal a consistent association between EGFR expression and response to Iressa in NSCLC patients.

Phase II clinical trials in several other tumours are underway. A study of Iressa monotherapy (500 mg/day) in patients with recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN), showed promising anti-tumour activity. The response rate was 10.6%, the disease control rate was 53% and median overall survival was 8.1 months. Toxicity was modest; skin toxicities (Grade 1/2) were observed in 48% of patients, and diarrhoea (grade 3 in three patients) was observed in 50% of patients (Cohen et al. 2003). In colon cancer, a phase II study (n=32) investigating combination therapy of Iressa (500 mg/day) with FOLFOX-4, a triple-combination standard treatment regimen for patients with advanced colorectal cancer, showed response rates of 75% for previously untreated patients and 23% for patients who had relapsed after chemotherapy. This compares well with historical response rates of 30%-55% and 9%, from the phase III trial programme of FOLFOX in first and second-line therapy, respectively. This combination therapy was generally well tolerated with the most common grade 3/4 adverse event being diarrhoea (Cho et al. 2003). In breast cancer, two phase II studies investigated Iressa monotherapy (500 mg/day) in patients with advanced disease. In one study, 10 of 31 patients (32%) had stable disease for at least 3 months (Baselga et al. 2003). In a second study, one of nine patients with acquired tamoxifen-resistant, oestrogen-receptor positive breast had a partial response and five had stable disease. One of 18 patients with oestrogen-receptor-negative breast cancer had a partial response and one patient had stable disease (Robertson et al. 2003).

3.3 Phase III Trials

The standard first treatment for patients with advanced NSCLC is combination chemotherapy with a platinum-based, two-drug regimen (e.g. cisplatin or carboplatin combined with gemcitabine, docetaxel or paclitaxel). Based on preclinical studies which showed that Iressa enhanced the efficacy of individual cytotoxic agents against a range of human tumour cell lines and xenografts-independent of the level of EGFR expression (Ciardiello et al. 2000; Sirotnak et al. 2000)-two phase III studies (Iressa NSCLC Trials Assessing Combination Treatment, INTACT 1 and 2) investigated Iressa combination with chemotherapy in more than 2,000 previously untreated NSCLC patients. Patients in INTACT 1 received cisplatin and gemcitabine, while patients in INTACT 2 received carboplatin and paclitaxel, and in both trials the chemotherapy was combined with Iressa 250 mg/day, Iressa 500 mg/day, or placebo. In both studies, addition of Iressa to chemotherapy failed to improve response rate, disease-free survival, or overall survival compared with chemotherapy given alone (Giaccone et al. 2004; Herbst et al. 2004). However, these placebo-controlled studies confirmed the favourable safety profile of Iressa reported in the IDEAL trials. Except for addition of dose-dependent diarrhoea and skin rash, which was less frequent in the 250-mg/day group, the toxicity profile was similar to that in patients receiving chemotherapy alone. The failure to translate the added benefit of combining Iressa and chemotherapy, anticipated from animal model studies (Ciardiello et al. 2000; Sirotnak et al. 2000), into the clinical setting in the INTACT trials is disappointing but should not be interpreted to mean that such combinations will be inappropriate in all settings (other stages of disease/other tumours/other agents). No animal studies were performed examining concurrent treatment with two cytotoxic agents and Iressa.

4 Future Prospects

Clinical trials with Iressa and other EGFR-targeted drugs (e.g. erlotinib/ Tarceva and cetuximab/Erbitux) have clearly demonstrated that this new approach to cancer therapy is of great benefit for some patients (Grunwald and Hidalgo 2003). However, it is equally clear that some patients in an unselected population with, for example, advanced NSCLC, do not derive significant benefits from treatment with this class of drugs. Unlike the case of breast cancer patients, where trastuzumab (Herceptin) treatment is determined by the presence or absence of overexpression of ErbB2, the very small number of clinical studies on linkage between tumour EGFR expression and response to EGFR-targeted therapies have so far failed to establish any correlation. Currently, the best indicator of response to gefitinib is symptom improvement, which typically occurs early during the course of treatment and is associated with tumour response, time to progression and overall survival (Cella et al. 2003). A huge challenge for the future is to identify either pathological or mechanism-based biomarkers which accurately predict the likelihood of therapeutic benefit from treatment with EGFR inhibitors. Formidable intellectual and technical barriers must be surmounted to achieve this objective. For example, to what extent would a complete knowledge of the expression or activity levels of all signal transducers, downstream of or linked to EGFR, help in patient stratification, and how might the best quantitative methods for putative biomarker measurement be defined and applied in the routine pathology laboratory setting? Measurement of EGFR expression by IHC presents major problems for routine application, since there is no consensus on methods for tumour sampling, storage, preparation and analysis. Many translational science laboratories are focussing their attention on the use of IHC methods to measure activated receptors [e.g. phosphorylated (p) EGFR and other ErbBs] and key downstream mediators like pMAPK and pAKT, as well as putative endpoints of drug action, like changes in cell cycle status (eg p27^{KlP1}), proliferative rate (eg Ki67/Mib1) and apoptosis (eg Tunel) in Iressa-treated patients (Albanell et al. 2001, 2002; Cappuzzo et al. 2003; Daneshmand et al. 2003; Mukohara et al. 2003; Rojo et al. 2003). Because of the inaccessibility to diagnostic biopsy sampling of many tumours, and the cost and complexity of biomarker analyses, the age of targeted drug treatment tailored to the (individual) tumour remains elusive. In immediate practical terms, the fact that Iressa is generally well tolerated means that there is no reason to exclude patients from treatment. Furthermore, as symptom improvement and tumour response generally occur soon after the start of treatment (IDEAL trials), the patients who do benefit from Iressa treatment will be rapidly identified.

In the longer term, the application of oncogenomic and/or proteomic analysis to tumour diagnostic samples may open a realistic prospect for individually targeted treatment. Important pilot studies in Iressa-treated xenograft models (Zembutsu et al. 2003) and patient samples have begun (Natale et al. 2003), and the increasing availability and use of frozen tumour tissue microarrays (Fejzo and Slamon 2001) hold great promise for the future.

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