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PROTEIN TYROSINE PHOSPHATASES: Structure and Function, Substrate Specificity, and Inhibitor Development

Zhong-Yin Zhang
Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461; e-mail: zyzhang@aecom.yu.edu

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■ **Abstract** Protein tyrosine phosphatases (PTPs) are signaling enzymes that control a diverse array of cellular processes. Malfunction of PTP activity is associated with a number of human disorders. Recent genetic and biochemical studies indicate that PTPs represent a novel platform for drug discovery. Detailed knowledge of PTP substrate specificity and the wealth of structural data on PTPs provide a solid foundation for rational PTP inhibitor design. This review summarizes a correlation of PTP structure and function from mutagenesis experiments. The molecular basis for PTP1B and MKP3 substrate recognition is discussed. A powerful strategy is presented for creating specific and high-affinity bidentate PTP inhibitors that simultaneously bind both the active site and a unique adjacent site. Finally, recent advances in the development of potent and selective inhibitors for PTP1B and Cdc25 are described.

INTRODUCTION

The precise and rapid propagation of cellular signaling events demands both strict and flexible regulatory processes. Protein phosphorylation is one of the major post-translational modification mechanisms that cells utilize to control various cellular regulatory processes. The addition or removal of a phosphoryl moiety from a protein can generate a recognition motif for protein-protein interactions, control protein stability, and most importantly, modulate enzyme activity. Approximately 30% of cellular proteins are phosphoproteins (1) and the majority of protein phosphorylation in eukaryotic cells occurs on serine or threonine residues. Tyrosine phosphorylation accounts for only 0.01% to 0.05% of the total protein phosphorylation (2). However, upon oncogenic transformation or growth factor stimulation, the level of tyrosine phosphorylation increases to 1%–2% of the total protein phosphorylation in the cell. Although tyrosine phosphorylation occurs to a much smaller extent, it has become clear that tyrosine phosphorylation is essential in controlling normal cellular growth, differentiation, metabolism, cell cycle, cell-cell communications,

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cell migration, gene transcription, ion channels, the immune response, and survival (3). Abnormal tyrosine phosphorylation is assoicated with many human diseases including cancers, diabetes, rheumatoid arthritis, and hypertension.

In vivo, tyrosine phosphorylation is reversible and dynamic; the phosphorylation states are governed by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Like PTKs, PTPs constitute a large family of enzymes (4, 5). PTPs can exert both positive and negative effects on a signaling pathway and play crucial physiological roles in a variety of mammalian tissues and cells (6). It is important to note, as observed with PTKs, deregulation of PTP activity can also contribute to the pathogenesis of many human diseases (7). For example, studies of PTP1B knockout mice show that the PTP1B is a major negative regulator of insulin signaling in muscle and liver, and the loss of PTP1B activity leads to enhanced insulin sensitivity and resistance to weight gain in mice (8, 9). The findings suggest the possibility of treating type 2 diabetes and/or obesity with inhibitors that block PTP1B activity. In addition to diabetes, PTPs have now been implicated in a wide variety of other disorders, including cancer (7). As a consequence, PTPs represent novel molecular targets for the development of medicinal agents that possess distinct modes of action. Clearly, a prerequisite for the successful development of potent and selective PTP inhibitors is a detailed understanding of the molecular basis of PTP catalysis and substrate specificity. In this review, I summarize our current understanding of PTP structure and function. I then discuss the molecular basis of PTP substrate specificity, focusing primarily on PTP1B and MAP kinase phosphatase 3. Finally, I highlight recent progress in developing small-molecule PTP inhibitors targeted to PTP1B and the cell cycle regulator Cdc25.

PTP STRUCTURE AND FUNCTION

The PTPs are a family of enzymes whose structural diversity and complexity rival those of the PTKs. Unlike PTKs, however, which share sequence identity with protein serine/threonine kinases, the PTPs show no sequence similarity with the protein serine/threonine phosphatases. PTPs are characterized by the presence of the active site signature motif $(H/V)C(X)_5R(S/T)$ in the conserved catalytic domain (10). In addition to the catalytic domain, PTPs are decorated with a wide range of structural elements including SH2 domains, PDZ domains, extracellular ligand binding domains, and many others (5). A recent estimate from the nearly completed human genome sequence suggested that humans have 112 PTPs (11), which include both the tyrosine-specific and dual-specific phosphatases. The tyrosine-specific phosphatases, such as PTP1B, hydrolyze phosphotyrosine (pTyr)-containing proteins, while the dual-specific phosphatases, such as Cdc25, can utilize protein substrates that contain pTyr, as well as phosphoserine (pSer) and phosphothreonine (pThr). The tyrosine-specific PTPs can be further categorized into two groups: receptor-like and intracellular. The receptor-like PTPs,

exemplified by CD45, generally have an extracellular putative ligand-binding domain, a single transmembrane region, and one or two cytoplasmic PTP domains. The intracellular PTPs, exemplified by PTP1B and SHP1, contain a single catalytic domain and various amino or carboxyl terminal extensions including SH2 domains that have targeting or regulatory functions. Examples of dual-specificity phosphatases include the MAP kinase phosphatases, the cell cycle regulator cdc25 phosphatases, and the tumor suppressor PTEN. All PTPs are characterized by their sensitivity to vanadate, ability to hydrolyze *p*-nitrophenyl phosphate, insensitivity to okadaic acid, and lack of metal ion requirement for catalysis.

The three-dimensional structures of over a dozen PTPs (mostly catalytic domains) have been determined. These structures have helped us understand PTP function at the molecular level by defining the interactions that stabilize the active site structure and substrate complex formation. The PTPs are α/β proteins with tertiary folds composed of a highly twisted mixed β-sheet flanked by αhelices on both sides (Figure 1). Despite the variation in amino acid sequences and the differences in substrate specificity, the crystal structures of the dual-specificity phosphatases display general folds that resemble the tyrosine-specific PTP structures. In addition, key structural features [e.g., the active site PTP loop formed by the (H/V)C(X)$_5$R(S/T) sequence motif and the general acid-containing surface loop] important for catalysis are conserved among all PTPs (12). The active site is located within a crevice ($\sim$9 Å deep for the tyrosine-specific PTPs and $\sim$6 Å deep for the dual-specificity phosphatases) on the protein surface. The much deeper active site pocket in the tyrosine-specific phosphatases selects exclusively pTyr-containing substrates (13, 14), while the more shallow active site cleft for the dual-specificity phosphatases may accommodate both pTyr and pSer/pThr (15, 16).

At the bottom of the active site is the phosphate-binding loop or P-loop formed by the PTP signature motif. The backbone of the P-loop creates a positive electrostatic potential owing to the outward arrangement of the side chains and the inward orientation of the partially positive-charged amide hydrogens. The bound phosphate is further stabilized by a closely positioned α-helix dipole, located amino-terminal to the P-loop, and the active site arginine. This design creates a complementary electrostatic binding-pocket for the phosphoryl moiety of incoming substrates. The active site is surrounded by several surface loops, which are important for catalysis and substrate recognition. In PTP1B, one of these loops, called the substrate recognition loop ($\alpha1-\beta1$ loop), contains Tyr46. A second loop contains the essential general acid/base Asp181. One important structural feature in PTPs is that ligand binding promotes a major conformational change of the general acid/base-containing loop, which brings Asp181 into the catalytic site and forms a hydrogen bond to the phenolic oxygen of pTyr to provide the general acid function (13, 17). In the ligand-bound form, the aryl side chain of Tyr46 and Phe182 sandwich the pTyr ring and delineate the boundaries of the pTyr-binding pocket in PTPs.

Site-directed mutagenesis combined with rigorous mechanistic studies on both tyrosine-specific and dual-specificity phosphatases have played a major role in

our understanding of the PTP catalytic mechanism (10). The PTPs employ covalent catalysis, utilizing the thiol group of the active site cysteine residue (Cys215 in PTP1B) as the attacking nucleophile, to form a thiophosphoryl enzyme intermediate (E-P) (18, 19). Substitutions of the Cys residue completely abrogate PTP activity (20). The nucleophilic cysteine is housed within the active site architecture specifically designed to bind a negatively-charged substrate. Thus, the microenvironment of the active site is largely responsible for the stabilization of the negative charge on the thiolate group (21). The E-P formation is assisted by a conserved aspartic acid (Asp181 in PTP1B) functioning as a general acid to neutralize the build-up of a negative charge on the leaving group (22, 23). For the hydrolysis of E-P, Asp181, which functions as a general acid in E-P formation, acts as a general base, abstracting a proton from the attacking water. This enhances the rate of E-P hydrolysis, regenerating the active enzyme (24, 25). The PTPs further accelerate the formation and hydrolysis of E-P by preferentially binding the pentacoordinated transition states with the guanidinium side chain of the active site arginine residue (Arg221 in PTP1B) (26, 27).

There is a rich literature describing the structure and function of PTPs based on kinetic analysis of various PTP mutants. To correlate structure and function, PTP amino acid residues that have been carefully examined are highlighted in Figure 1. The effects of mutation in the context of structural data are summarized in Table 1. Recent site-directed mutagenesis experiments in combination with detailed kinetic analysis provide further insight into PTP catalysis. For example, substitutions of residues in the general acid loop or the active site Arg cause perturbation of the exact positioning of the general acid in PTPs and impair its ability to protonate the leaving group (28–30). In addition, kinetic analysis of the Gln262 mutants suggest that the precise placement of both the general base and the water nucleophile are essential for optimizing E-P hydrolysis (28, 31). These results indicate that general acid/base catalysis in PTPs is coupled to the correct positioning of the Asp181-containing flexible loop, which is determined by proper interactions between the active site Arg and the conserved Trp at the hinge position. Collectively, as can be seen in Table 1, invariant and conserved residues are generally found to be important for either catalysis or structural integrity common to all PTPs. Studies on variable residues are just beginning, and it is likely that these residues will contribute to PTP substrate specificity and regulation.

PTP Substrate Specificity

As described above, extensive biochemical and structural studies on PTPs have led to a detailed understanding of the mechanism by which PTPs catalyze phosphate monoester hydrolysis. In spite of the remarkable progress in the identification and characterization of new PTPs and in the understanding of PTP catalysis, the molecular basis by which PTPs distinguish and recognize the diverse substrates they encounter in the cell remains largely undefined. This is partly because the physiological substrates for most PTPs are unknown. Furthermore, since the

TABLE 1 Correlation between effect of mutagenesis and structure/function of PTPs

Residue	Conservation	Effect of mutation	Structure/Function
Tyr46	Conserved	800-fold decrease in E-P formation	Hydrophobic packing with pTyr in the substrate
Arg47	Variable	10-fold decrease in E-P formation, no effect on E-P hydrolysis and *p*NPP reaction	Flexible, adopts two conformations for peptide recognition
Asp48	Conserved	20-fold decrease in E-P formation, no effect on E-P hydrolysis and *p*NPP reaction	Forms two H-bonds with the main chain N atoms of pTyr and the +1 position of peptide substrates
Glu115	Invariant	100-fold decrease in k_{cat}	Coordinates Arg221 with two H-bonds
Trp179	Invariant	Similar effects to those observed for the Asp181 mutants	Located at one of the hinges of the general acid loop, important for precise positioning of Asp181
Asp181	Invariant	10^3–10^4-fold decrease in E-P formation,10^1–10^2-fold decrease in E-P hydrolysis	General acid in E-P formation, general base in E-P hydrolysis
Phe182	Variable	10–20-fold decrease in both E-P formation and E-P hydrolysis	Hydrophobic stacking with pTyr in the substrate, important for precise positioning of Asp181
His214	Invariant	Increase in the pKa of the active site thiol group, 100-fold decrease in k_{cat}	The side chain of His214 interacts with the carbonyl of Cys215
Cys215	Invariant	Destroys phosphatase activity, retains substrate binding ability	Active site nucleophile for phosphate ester hydrolysis
Arg221	Invariant	10^4-fold decrease in k_{cat}, 10-fold increase in K_m when replaced with residues other than lysine (Arg to Lys substitution can restore K_m but not k_{cat}). Also affects the functionality of Asp181	Forms bidentate hydrogen bonds with the nonbridge phosphoryl oxygens in the substrate. Also interact with Trp179 in the general acid loop

(*Continued*)

TABLE 1 (*Continued*)

Residue	Conservation	Effect of mutation	Structure/Function
Ser222	Conserved	<10-fold decrease in E-P formation, 10^1–10^2-fold decrease in E-P hydrolysis	H-bond to stabilize the active site thiolate in E-P hydrolysis
Arg254	Invariant	50-fold decrease in k_{cat}, can be replaced by a Lys	Surfaced exposed, participates in a H-bond array to stabilize the P-loop
Arg257	Invariant	10^3-fold decrease in k_{cat}	Buried, H-bonds with carbonyls of residues in the P-loop, structural role
Gln262	Invariant	10-fold decrease in E-P formation, 100-fold decrease in E-P hydrolysis, confers phospho-transferase activity	Coordinates scissile oxygen in the substrate and the attacking water
Gln266	Invariant	10^2-fold decrease in E-P formation, 10-fold decrease in E-P hydrolysis	Makes H-bonds with the phosphoryl group and active site waters

physiological substrates for PTPs are phosphoproteins, even if the identity of the true physiological substrate is known, it remains a major technical challenge to obtain quantities of specifically and stoichiometrically phosphorylated proteins required for detailed enzymological studies. Thus, studies on PTP substrate specificity rely on the use of synthetic phospho-peptides that correspond to the phosphorylated species found in vivo. Based on available biochemical data, it appears that members of the PTP family display a spectrum of substrate specificity. In general, the tyrosine specific phosphatases show only moderate specificity in the context of peptide substrates, while the dual-specificity phosphatases exhibit more stringent substrate specificity. For example, at one end of the spectrum, PTP1B possesses extraordinary plasticity in accommodating various pTyr-containing sequences, while at the other end of the spectrum, MAP kinase phosphatase 3 (MKP3) displays exquisite substrate specificity, which requires extensive protein-protein interactions away from the active site for substrate recognition. In the following, the discussion on PTP substrate specificity focuses on PTP1B and MKP3.

Molecular Basis of Phospho-Peptides Recognition by PTP1B

An early study showed that PTP1B displayed limited selectivity toward peptides derived from in vivo phosphorylation sites (32). The k_{cat}/K_m (substrate specificity

constant) values for some peptide substrates approach the diffusion limit and are 3–4 orders of magnitude higher than that of pTyr, which suggests that residues flanking the pTyr moiety make important contributions to PTP1B substrate recognition. This study identified DADEpYLIPQQG, which corresponds to residues 988–998 in EGF receptor, as the best substrate for PTP1B ($k_{cat}/K_m = 2.88 \times 10^7\ M^{-1}\ s^{-1}$). This peptide was subjected to a classical alanine scanning in order to identify amino acids important for PTP1B recognition (33). The kinetic analysis revealed that PTP1B displayed a moderate preference for acidic residues at positions N-terminal to pTyr, particularly in the −1 position. Furthermore, in the context of the EGF receptor peptide, the hexapeptide DADEpYL-NH_2 was found to be an excellent substrate for PTP1B with a k_{cat}/K_m of $2.24 \times 10^7\ M^{-1}\ s^{-1}$ (34).

The crystal structure of DADEpYL-NH_2 bound to the catalytically inactive PTP1B/C215S revealed key features for peptide binding (13). Besides crucial hydrogen bonds and hydrophobic interactions between the pTyr residue and PTP1B active site, several hydrogen bonds involve the peptide backbone that stabilizes the binding interface. Only limited interactions involved peptide side chains, including interactions between the peptide carboxyl group of Glu and Asp in −1 and −2 positions with the guanidinium group of Arg47. This complex provides a structural interpretation for PTP1B's preference for N-terminal acidic residues and for the superiority of DADEpYL-NH_2 over pTyr.

From these limited studies, it appears that efficient substrate recognition by PTP1B requires the presence of amino acids on both sides of pTyr. However, because of the differences in sequence and size of the individual peptides examined, it is difficult to draw any definitive conclusions regarding the structural requirements for substrate recognition. Clearly, the use of peptide libraries may be more suitable for the elucidation of structural features that control substrate specificity for individual PTPs. Indeed, combinatorial peptide libraries have been useful in the determination of optimal amino acid sequence for protein kinase and SH2 domain recognition. Several studies on PTP1B specificity using combinatorial libraries have been described and provide interesting insights into the plasticity of PTP1B-phosphopeptides interaction.

An affinity-selection strategy was utilized to identify high-affinity peptides for PTP1B, which contained the nonhydrolyzable pTyr mimic, phosphonodifluoromethyl phenylalanine (35). This study found a clear preference for acidic residues Glu or Asp for all four positions, N-terminal to pTyr. In addition, position −1 could also accept aromatic residues such as Tyr and Phe with affinity comparable to substitutions by Glu and Asp. The position immediately C-terminal to pTyr (+1) did not show a strong preference, and residues Gly or Met appeared to be somewhat selected. The study also showed that the basic residue Arg was disliked in any position.

Another study (36) focused on the N-terminal positions −3 to −1 using a targeted peptide library containing the nonhydrolyzable pTyr mimic malonyltyrosine and confirmed the preference for Asp and Glu in position −2. However, these authors found an unexpected selectivity for aromatic residues in positions −3 and −1. Indeed, there was an obvious preference for Trp, Phe, and Tyr over Glu or

Asp at position −1. Position −3 appeared to accept various different substitutions equally well, discriminating only poorly between Asp, Glu, Gly, Trp, and hydroxyproline. Again, basic residues were unfavorable for substitution.

The preference for aromatic residues in position −1 was also seen in an "inverse alanine scanning" approach (37). In this method each Ala residue in the parent peptide Ac-AAAApYAAAA-NH_2, is separately and sequentially replaced by the 19 non-Ala amino acids to generate a library of 153 well-defined peptides. The relatively small number of peptides allows the acquisition of explicit kinetic data for all library members, thereby furnishing information about the contribution of individual amino acids with respect to substrate properties. Results from this study showed that PTP1B can accommodate acidic (Glu/Asp), aromatic (Phe/Tyr), and hydrophobic (Leu) residues at the −1 position. A preference for Leu at the −3 position was observed, as well. This study also scanned the four C-terminal positions and found a clear preference for Met in +1. In addition, acidic residues Glu and Asp were selected for the +2 position, and a strong preference for aromatic residues Phe and Tyr in the +3 position was observed. In general, a strong nonpreference for Lys and Arg residues in any position was also apparent. The deduced consensus peptide ELEFpYMDYE is a highly potent PTP1B substrate with a k_{cat}/K_m of $2.2 \pm 0.05 \times 10^7$ M^{-1} s^{-1}, which is comparable to the EGF receptor peptide DADEpYLIPQQG. This is remarkable because the two peptides differ significantly in size and amino acid sequence.

The results from the combinatorial library studies described above used experimentally very different approaches to assess the specificity of PTP1B for phosphopeptide recognition and led to similar results. This increases the confidence in the results of each study. Collectively, these studies suggest that many substitutions are possible at several positions adjacent to the pTyr and that there may not be a single "true" consensus sequence for PTP1B. For instance, the studies indicate that peptides with acidic or aromatic residues at the −1 position are comparable in their binding to PTP1B. Furthermore, considerable data in the literature suggests that PTP1B is somewhat promiscuous in its substrate preference as it dephosphorylates a wide variety of protein/peptide substrates, and PTP1B hydrolyzes several unrelated pTyr-containing peptides with almost equal efficiency as the EGF receptor peptide (38). This raises the question: how is it possible that peptides with quite different amino acid sequence can bind equally well to PTP1B?

The structural basis for the plasticity of PTP1B substrate recognition was revealed by a comparison of the structure of PTP1B-DADEpYL-NH_2 with two novel structures of PTP1B with the peptides ELEFpYMDYE and DAD(Bpa)pYLIPQQG, respectively, containing aromatic amino acids [Phe and *p*-benzoylphenylalanine (Bpa)] in position −1 (38). In all three structures, the bound peptide is similarly oriented and maintains the same N- to C-terminal direction relative to PTP1B surface. The dominant interactions involve pTyr itself with PTP1B. In addition, the backbone of certain positional equivalent residues is in similar conformations in all three bound peptides. All three structures maintain two conserved hydrogen bonds between the Asp48 carboxylate side chain and the main chain nitrogens

of the pTyr and the +1 residue, and a third between the main chain nitrogen of Arg47 and the main chain carbonyl of the −2 residue. Consequently, the peptide backbone of residues from the −1 to the +1 positions in all three structures adopts a twisted β-strand conformation, which ensures that the pTyr is bound to PTP1B in an identical manner, regardless of the specific sequence adjacent to it.

The ability to accommodate both acidic and hydrophobic residues immediately N-terminal of pTyr appears to be conferred upon PTP1B by a single residue, Arg47. Depending on the nature of the N-terminal amino acids, the side chain of Arg47 can adopt two different conformations, generating two sets of distinct peptide binding surfaces. When an acidic residue is positioned at the −1 position, a preference for a second acidic residue is observed at the −2 position. However, when a large hydrophobic group occupies the −1 position, Arg47 adopts a new conformation so that it can participate in hydrophobic interactions with both the −1 and −3 positions. Thus the conserved mode of interactions between the peptides and PTP1B and the unique peptide sequence–specific conformational changes in PTP1B provide a structural basis for the observed plasticity in PTP1B substrate recognition.

Functional Significance of PTP1B Active Site Plasticity

The ability of PTP1B to recognize common substrate features and to generate altered binding surfaces to accommodate diverse amino acid sequences may be essential for its function as a broad specificity PTP. Numerous studies have implicated PTP1B in various signaling pathways. For example, PTP1B has been suggested as a negative regulator of insulin signaling (8, 9, 39–41). Both insulin receptor and insulin receptor substrate-1 have been suggested to be direct substrates for PTP1B (8, 9, 42, 43).

In addition to a role in insulin signaling, PTP1B is overexpressed in association with the expression of $p185^{c\text{-}erbB\text{-}2}$ in human breast and ovarian cancers (44, 45). PTP1B is capable of suppressing transformation by Neu (46), v-Crk (47), v-Src (48), and v-Ras (47). Recently, PTP1B has been identified as the major PTP that dephosphorylates and activates c-Src in several human breast cancer–cell lines (49). PTP1B is also capable of antagonizing signaling by the EGF receptor (50, 51) and the oncoprotein $p210^{bcr\text{-}abl}$ (52) by dephosphorylating directly the EGF receptor and the $p210^{bcr\text{-}abl}$ tyrosine kinase. Furthermore, PTP1B can negatively regulate integrin-mediated adhesion and signaling by binding and dephosphorylating β-catenin (53) and $p130^{cas}$ (Crk associated substrate) (54). Interestingly, other potential substrates for PTP1B include the prolactin-activated signal transducers and activators of transcription STAT5a and STAT5b (55). These results, taken together, suggest that PTP1B may be a participant in several pathways.

It is proposed here that both precise cellular compartmentalization/localization and active site plasticity are required for PTP1B to accomplish its proposed in vivo functions. It is important to note that many of the suggested PTP1B substrates are receptor tyrosine kinases (e.g., insulin receptor and EGF receptor) and adapter

proteins (e.g., insulin receptor substrate-1 and p130^{cas}) that contain multiple tyrosine phosphorylation sites. A choice that a cell has to make is whether to enlist multiple PTPs that are highly specific for each phosphorylation site or to rely on a broad specificity PTP, such as PTP1B, that is strategically positioned in the cell for efficient substrate dephosphorylation. Protein phosphorylation sites are generally located on surface loops, which may be mimicked by peptides. Because the k_{cat}/K_m values of peptide substrates for PTP1B already approach the efficiency limited by diffusional events, it is unlikely that protein substrates for PTP1B will exhibit kinetic properties that are any better than the peptide substrates. Furthermore, although the question whether PTP1B cooperates with other PTPs in the dephosphorylation of its targets remains unanswered, recent studies indicate that PTP1B catalyzed dephosphorylation of insulin receptor, and insulin receptor substrate-1 does not display any site specificity (42, 43). Thus, it is possible that cellular localization may dictate the substrate accessibility for PTP1B. Once the substrate is exposed to PTP1B complete dephosphorylation occurs. Indeed, considering the large number of PTKs and their substrates, many of which have multiple phosphorylation sites, it is unrealistic to expect every PTP (total number of tyrosine specific PTPs estimated to be 50) to be specific for a single phosphorylation site.

PTP1B is localized to the cytoplasmic face of the endoplasmic reticulum (ER) through the C-terminal 35 residues (48, 56). Many receptor protein tyrosine kinases (RPTKs) are internalized after activation. In addition, newly synthesized RPTKs need to be in unphosphorylated states (to avoid unwanted phosphorylation) in transport from the ER to the plasma membrane. Thus, it may be necessary for the cell to place a somewhat nonspecific PTP in the proximity of the RPTKs in order to inactivate the kinases efficiently and to prevent the premature activation of the kinases. Indeed, Lammer and colleagues found that various RPTKs, located at different cellular compartments, exhibited different susceptibilities to dephosphorylation by PTPs and that these differences were based on the cellular location of the PTPs (57). Transient overexpression of PTP1B and a panel of RPTKs showed that PTP1B preferentially dephosphorylates the receptor precursors likely localized in the ER, which is in accord with the known cellular localization of PTP1B. PTP1B also contains a proline-rich SH3 domain-binding motif, which may be required for it to bind the Src kinase in the cytoplasmic membrane or p130^{cas} in the focal adhesion complexes (54). Given the extensive contiguous nature of the plasma membrane, the endosomal apparatus, the ER, and the focal adhesion complexes and cytoskeleton, it is conceivable that PTP1B is able to come in contact with a number of cellular substrates. Because of its broad specificity, PTP1B can cleave the phosphoryl moiety from multiple and diverse autophosphorylation sites in RPTKs with high efficiency.

However, it is crucial to understand that the PTP family consists of a diverse group of enzymes, which exhibit a wide spectrum of substrate recognition characteristics. Unlike PTP1B, certain members of the PTP family are very specific. For example, the dual-specificity phosphatases Cdc25 and MKP3 display rigid substrate specificity, capable of recognizing only their native phosphorylated

substrates, the cyclin-dependent kinases (CDKs) and the extracellular signal-regulated kinases (ERKs). Small-molecule substrates, peptides, and even denatured physiological substrates are poorly dephosphorylated, which indicates substantial involvement of specific protein-protein interactions between the phosphatases and their corresponding protein substrates. The kinase-associated phosphatase (KAP), which is a member of the dual-specificity group of PTPs, dephosphorylates phospho-Thr160 in the CDK2 activation loop (58). The crystal structure of KAP in complex with phosphoCDK2 reveals that the major interactions between the two molecules reside in the C-terminal lobe of CDK2 and the C-terminal helix of KAP, regions that are remote from the CDK2 activation loop and KAP active site (59). This provides a nice example that structural features outside the immediate vicinity of the phospho–amino acid site in a substrate play an important role for the high specificity of a PTP. In the following sections, I discuss some of the recent studies on the molecular basis of MKP3 substrate specificity.

Mechanism of MKP3 Substrate Recognition

The MKPs are dual-specificity phosphatases capable of dephosphorylating both pTyr and pThr in the activation loop of MAP kinases. Previous studies suggest that these MKPs display distinct in vivo substrate preferences for the various MAP kinases. For example, MKP3 is highly specific in deactivating ERK1/2. MKP3 exhibits very low activity toward small aryl phosphates (e.g., for *p*NPP and pTyr, $k_{cat}/K_m = 2.0\ M^{-1}\ s^{-1}$) (60) and the bisphosphorylated peptide derived from the activation lip of ERK2 (DHTGFLpTEpYVATR, $k_{cat}/K_m = 5.0\ M^{-1}\ s^{-1}$) (61). In addition, MKP3 can dephosphorylate only pTyr but not pThr in the context of the synthetic bisphosphorylated ERK2-derived peptide. In contrast, kinetic analysis with the physiological substrate, bisphosphorylated ERK2, showed that the k_{cat}/K_m for the MKP3-catalyzed hydrolysis of ERK2/pTpY ($3.8 \times 10^6\ M^{-1}\ s^{-1}$) is 10^6-fold higher than those for the hydrolysis of *p*NPP or the ERK2-derived phosphopeptide (62). Furthermore, MKP3 is capable of dephosphorylating efficiently both the pTyr and the pThr on ERK2. Given the huge difference in intrinsic chemical reactivity (10^5-fold) between pTyr and pThr, it is remarkable that MKP3 can hydrolyze pThr and pTyr in ERK2 with similar efficiency. What is the molecular basis for the strikingly high substrate specificity and dual specificity of MKP3?

Structural and biochemical studies suggest that a conformational reorganization in the active site is required for MKP3 to achieve full activity (16, 60, 63). The crystal structure of the catalytic domain of MKP3 in the absence of its substrate reveals a distorted active site structure incompetent for catalysis (16). Unlike other PTPs, the MKP3 active site residues (Cys293 and Arg299) are misaligned, and the general acid (Asp262) is positioned away from the active site. Binding of ERK2 to MKP3 triggers a powerful activation of MKP3 phosphatase activity toward *p*NPP (64). Kinetic evidence suggests that ERK2 binding to MKP3 elicits activation of MKP3 activity by facilitating the repositioning of active site residues and general acid loop closure in MKP3 (60). Binding of a small peptide substrate or a

nonspecific substrate to MKP3 may not be sufficient to enable such a conformational change in MKP3 so that the general acid Asp262 will not function in catalysis. This explains why MKP3 is unable to hydrolyze pThr and exhibits low activity toward the ERK2-derived peptide.

The molecular basis for efficient dual dephosphorylation of ERK2/pTpY by MKP3 may lie in the specific interactions between ERK2 and MKP3 that are not possible between nonspecific substrates and MKP3. The 10^6-fold higher activity of MKP3 for ERK2/pTpY, as compared to that for the ERK2-derived phosphopeptide harboring the same pTEpY motif, indicates that there is no structural complementarity between the peptide substrate and MKP3 and that the peptide by itself is insufficient to induce full activation of MKP3. This also suggests that structural features outside the immediate vicinity of the pTEpY site of ERK2 play an important role for the high specificity of MKP3 for ERK2. Indeed, recognition and activation of MKP3 by ERK2 involves multiple regions of MKP3, some of which are important for high-affinity binding whereas others are more important for the ERK2-induced MKP3 activation (65). Thus, substrate specificity of MKP3 is linked to the ability of the substrate to induce productive orientation in the active site, which provides a powerful mechanism to ensure high fidelity in ERK2 kinase inactivation.

PTP INHIBITOR DEVELOPMENT

Because balanced protein tyrosine phosphorylation is critical for the maintenance of cellular homeostasis, it is not surprising that malfunction of PTPs has been linked to many human diseases (7). Consequently, in those instances where PTP activity is inappropriately high, PTP inhibitors may constitute a valuable new family of therapeutic agents. Drug development targeted to protein phosphatases has already yielded successes. For example, cyclosporin and FK506, inhibitors of the protein Ser/Thr phosphatase calcineurin, are among the most effective immunosuppressive drugs used after organ transplantation. However, treating disease with PTP inhibitors was not seriously considered until recently. Two major issues, both of which relate to specificity, are responsible for the hesitation in developing PTP-based therapy. There is concern that because the PTP active site (pTyr binding site) is highly conserved, it would be impossible to obtain selective PTP inhibitors. There is also concern that a PTP may regulate multiple signaling pathways while at the same time, a single pathway may be controlled by several PTPs. Thus, PTP inhibition may give rise to unwanted side effects. These are valid concerns, and significant progress has been made that is beginning to alleviate these concerns.

As discussed above, PTP1B is ubiquitously expressed and is implicated in a variety of signaling processes. Thus, PTP1B may not be considered an ideal target for drug development. However, PTP1B$^{-/-}$ mice show increased insulin receptor and insulin receptor substrate-1 phosphorylation and enhanced sensitivity to insulin in skeletal muscle and liver (8, 9). In addition, PTP1B$^{-/-}$ mice have remarkably low adiposity and are protected from diet-induced obesity. Perhaps most

importantly, these mice appeared to be normal and healthy, which indicates that specific PTP1B inhibitors may be free of side effects and have selective therapeutic efficacy (antidiabetes/obesity) even though PTP1B is expressed ubiquitously. These studies provide the first proof-of-concept for developing PTP-based therapeutics. Furthermore, small molecules designed to inhibit PTPs not only have promise as pharmaceutical agents but also function as probes for elucidating the roles of PTPs in specific intracellular pathways involved in normal cellular processes and in the pathogenesis of certain diseases. It is not surprising, therefore, that interest in the development of PTP inhibitors has intensified in recent years. Most of the research effort on PTP inhibitor development has been focused on PTP1B and the cell-cycle regulator Cdc25 phosphatases. Several potent and selective small-molecule inhibitors have been described for PTP1B and Cdc25.

PTP1B Inhibitors

Although pTyr by itself binds weakly to PTP1B, pTyr is essential for binding of peptide/protein-based substrates by PTP1B (32, 66). In fact, the recognition pocket for pTyr represents the dominant driving force for peptide binding since pTyr contributes about 53% of the peptide solvent-accessible surface area (13). Since high-affinity substrate binding also requires amino acids flanking the pTyr (33, 34), one approach toward the design of potent and selective PTP1B inhibitors relies on the incorporation of a nonhydrolyzable analog of pTyr into specific optimal peptide templates. Several nonhydrolyzable analogs of pTyr have been prepared and inserted into PTP1B-targeted peptides (67).

The most commonly used phosphorus-based pTyr analog is phosphonodifluoromethyl phenylalanine (F_2Pmp) (68). When F_2Pmp replaces the pTyr in the hexapeptide DADEpYL-NH_2, the K_i for the resulting peptide bearing F_2Pmp (200 nM for PTP1B) is over 1000 times more potent than the same peptide containing phosphonomethyl phenylalanine (Pmp) (69, 70). This has been attributed to a direct interaction between the fluorine atoms and PTP1B active site residues (69). The enhanced affinity of F_2Pmp over Pmp likely arises from the ability of the fluorine atoms in F_2Pmp to interact with the PTP1B active site residues in a fashion analogous to that involving the phenolic oxygen and side chains in the active site of PTP1B (10, 71).

However, owing to proteolytic susceptibility and weak partitioning across the plasma membrane, peptide-based compounds are not highly desirable for the development of medicinally effective drugs. In addition, there are also limitations of using peptides as platforms for pTyr mimetics incorporation (72). In short, small-molecule pTyr mimetics that exhibit higher than or comparable to that of pTyr may not yield a potent PTP1B inhibitor when introduced into a peptide template, owing to the need to maintain peptide binding outside of the pTyr pocket and the less than ideal interactions between the pTyr analog and the active site. Thus, recent efforts have centered on the development of selective, low molecular weight nonpeptidic PTP1B inhibitors.

As discussed above, studies have shown that invariant PTP residues are either involved in catalysis or form the pTyr binding site, which indicates that PTPs utilize similar strategies for phosphomonoester hydrolysis and pTyr recognition. Can specificity be achieved as one targets PTP active site for inhibitor development? A similar question was raised in the protein kinase field because of the structural conservation of the kinase active site, especially the ATP binding pocket. Inhibitors that target the peptide/protein substrate-binding surface generally produced less potent inhibitors than those that exploit the ATP binding pocket (73). Despite the structural conservation of the ATP binding pocket, many specific kinase inhibitors have been obtained that compete with ATP binding (74). Structural studies reveal that specificity comes from the fact that only a portion of each inhibitor interacts with the residues that bind ATP, whereas the rest of the molecules make contact with other residues situated outside the ATP-binding pocket.

It is known that pTyr alone is not sufficient for high-affinity binding by PTPs and residues adjacent to pTyr contribute to efficient substrate recognition. Furthermore, the pTyr binding site in PTPs is obviously smaller than the ATP site in protein kinases. Thus for PTP inhibitor design, it is even more critical to consider adjacent peripheral sites in addition to the active site in order to gain potency and selectivity. As a first step toward the development of selective, low molecular weight nonpeptidic PTP1B inhibitors, active site substrate specificity was investigated to identify small-molecule substrates that display kinetic properties comparable to those of peptides. Although nonpeptidic aryl phosphates are in general much poorer substrates than the pTyr-containing peptides, appropriately functionalized aromatic phosphates can exhibit K_m values in the low μM range and are hydrolyzed by PTP1B as efficiently as the best peptide substrates reported for this enzyme (75). For example, bis-(*para*-phosphophenyl) methane (BPPM) was one of the best low–molecular weight nonpeptidic substrates identified for PTP1B ($k_{cat} = 6.9\,s^{-1}$, $K_m = 16\ \mu M$).

The crystal structure of PTP1B/C215S complexed with BPPM showed that BPPM binds, as expected, at the pTyr site (the active site) and provided structural explanations for the higher affinity of BPPM relative to pTyr (76). Quite unexpectedly, the crystal structure revealed the presence of a second aryl phosphate-binding site positioned adjacent to the active site. This second site lies within a region not conserved among PTPs. As a consequence, this unanticipated observation has suggested a new paradigm for the design of bidentate potent and specific PTP1B inhibitors that bind both the active site and a unique adjacent peripheral site. It is proposed that PTP1B inhibitors able to simultaneously occupy both the active site and the second site should exhibit enhanced affinity and specificity. The rationale for this is based on the principle of additivity of free energy of binding as illustrated in Figure 2. Furthermore, the interaction of an inhibitor with two independent sites (e.g., pTyr site and site X in Figure 2) on PTP1B would be expected to confer exquisite specificity, because other PTPs do not possess an identical second site interaction.

Using this strategy, several bis-aryldifluorophosphonate inhibitors that display reasonable selectivity for PTP1B have been obtained (77–79) (Figure 3). Recently,

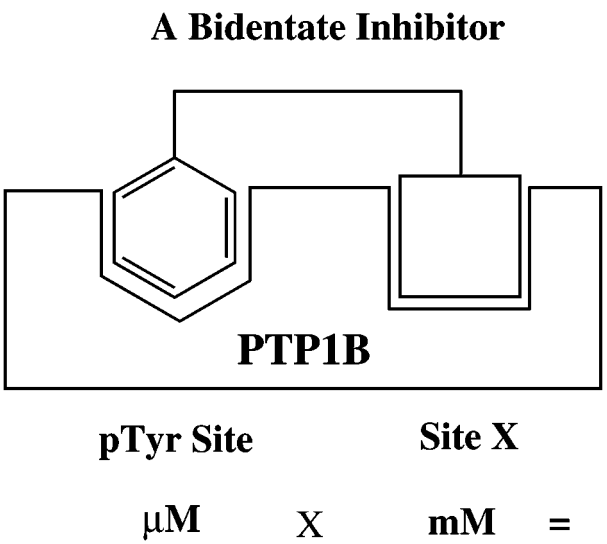
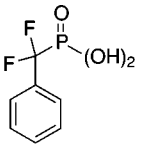


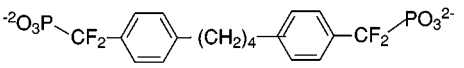
Figure 2 A strategy for creating selective and high-affinity PTP1B inhibitors. Based on the principle of additivity of free energy of binding, high-affinity ligands can be obtained by linking two functional groups that bind to the active site (pTyr binding site) and a peripheral site X. Specificity arises from the fact that site X is not conserved and from the fact that the tethered ligand has to bind both sites simultaneously.

a combinatorial library was prepared that led to the identification of the most potent and selective PTP1B inhibitors (Compound 5) to date (80). Compound 5 has a K_i of 2.4 nM against PTP1B and displays orders of magnitude selectivity in favor of PTP1B against a panel of PTPs. These results suggest that it is possible to achieve selectivity in PTP1B inhibitor design. Although the difluorophosphonate functionality, which serves as an excellent nonhydrolyzable mimetic of the phosphate moiety, will likely interact in the desired inhibitory fashion with all PTPs, the molecular scaffolds to which the difluorophosphonate is attached render the inhibitors PTP1B-selective.

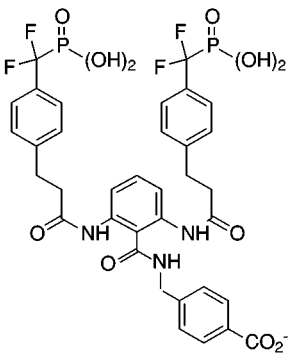
There is no question that potent PTP1B inhibitors can be obtained when the difluorophosphonate moiety is incorporated into an appropriate template. However, there is concern that the dianionic nature of the phosphonate group may compromise its ability to cross cell membranes. Recent studies suggest that this may not be a serious problem because phosphonate-containing Src SH2-domain ligands penetrate cell membranes (81). In addition, some difluorophosphonate PTP1B inhibitors display insulin mimetic activity in vivo (Z-Y. Zhang, unpublished results). On the other hand, since the PTP active site is designed to bind phosphate, there is concern that elimination of the negative charges from the phosphonate may decrease its affinity for the PTP. Thermodynamic studies of ligand binding to PTP1B and its substrate-trapping mutants showed that PTP1B/C215S displays enhanced enthalpic contribution for the binding of negatively charged ligands



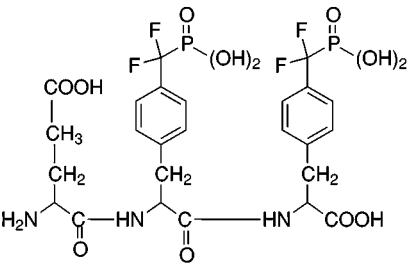
1, α,α-difluorobenzyl phosphonic acid, K_i = 2,500 μM, ref. 90



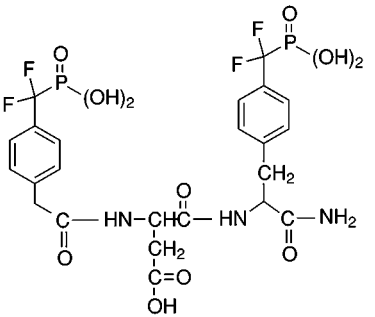
2, IC_{50} = 4.4 μM, ref. 77



3, K_i = 0.93 μM, ref. 78



4, IC_{50} = 0.04 μM, ref. 79

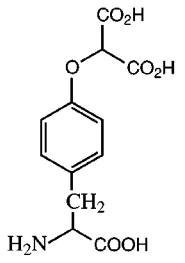


5, K_i = 2.4 nM, ref. 80

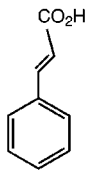
Figure 3 Structures of difluorophosphonate-containing PTP1B inhibitors.

(e.g., F_2Pmp-containing peptides, suramin, and vanadate), possibly because of the removal of the repulsive interaction between the active site thiolate anion and the negatively charged phosphate mimics (70). Consequently, a properly functionalized phosphate surrogate with less or no negative charge would be expected to bind strongly to the native PTP1B active site when attached to an appropriate aromatic framework.

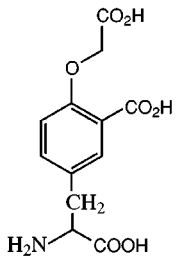
To enhance cellular penetration and provide additional options (e.g., prodrug protection) for inhibitor design, several nonphosphorus–based carboxylate-containing pTyr surrogates are being evaluated (Figure 4). These include O-malonyltyrosine (82), cinnamic acid (83), 3-carboxy-4-(O-carboxymethyl) tyrosine (84), salicylic acid and benzoic acid (85), 2-(oxalylamino)-benzoic acid (86), and 5-carboxy-2-naphthoic acid (87). Like pTyr, none of these pTyr mimetics exhibit high affinity toward PTP1B. However, when attached to an appropriate scaffold,



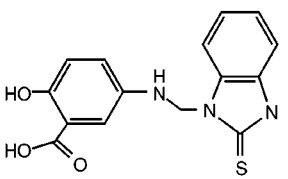
6, O-malonyltyrosine
Ref. 82



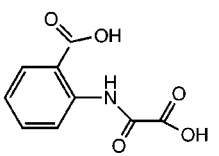
7, cinnamic acid, ref. 83



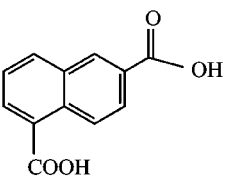
8, 3-carboxy-4-(O-carboxymethyl) tyrosine, ref. 84



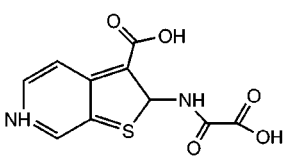
9, salicylic acid derivative
K_i = 61 μM, ref. 85



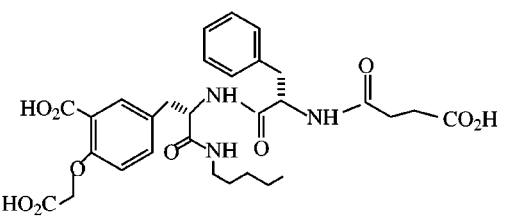
10, 2-(oxalylamino)-benzoic acid, K_i = 200 μM, ref. 86



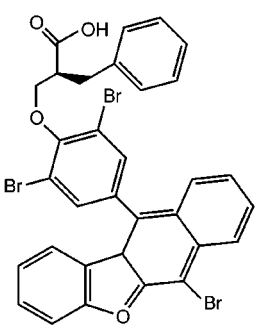
11, 5-carboxy-2-naphthoic acid, K_i = 46 μM, ref. 87



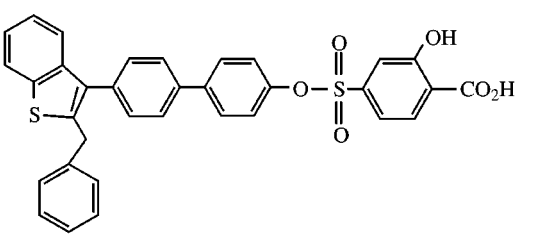
12, K_i = 5.1 μM, ref. 91



13, K_i = 0.25 μM, ref. 89



13, IC_{50} = 89 nM, ref. 92



14, IC_{50} = 28 nM, ref. 88

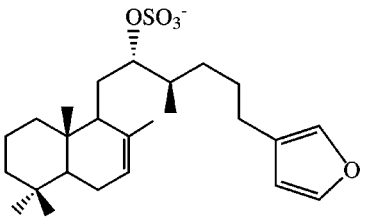
Figure 4 Structures of nonphosphorus small-molecule PTP1B inhibitors.

the pTyr surrogate–containing compounds can be very effective PTP1B inhibitors. Crystal structures of PTP1B with many of the nonphosphorus inhibitors revealed that they are active-site directed (86, 88, 89). Increasing the size and hydrophobicity of the aromatic ring to which the phosphate mimic is attached generally leads to enhanced affinity (75, 85, 90). Beginning with the crystal structure of PTP1B bound with 2-(oxalylamino)-benzoic acid (10), a competitive inhibitor of PTP1B with a K_i value of $\sim$200 μM, Iversen and colleagues introduced chemical modifications to Compound 10 and generated Compound 12 with enhanced PTP1B affinity ($K_i = 5.1$ μM) (91). Extensive modification of the 3-carboxy-4-(O-carboxymethyl) tyrosine core (Compound 8) led to several small-molecule peptidomimetics (e.g., Compound 13) that displayed submicromolar potency against PTP1B and augmented insulin action in the cell (89). Variation on the themes of benzyloxyacetic acid and salicylic acid produced Compounds 13 and 14, which normalized plasma glucose and insulin levels in the ob/ob and db/db mouse models (88, 92). In principle, an identical approach (i.e., to create bidentate inhibitors that bind both the active site and a unique adjacent peripheral site) could also be used to produce unique low molecular weight nonphosphorus compounds specific for individual PTP isoenzymes.

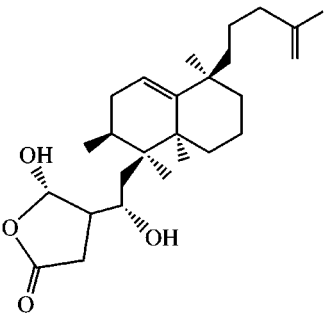
Cdc25 Inhibitors

The Cdc25 phosphatases play an important role in cell-cycle regulation by removing inhibitory phosphates from tyrosine and threonine residues of cyclin-dependent kinases. There is strong evidence indicating Cdc25A and Cdc25B as potential oncogenes (93, 94), and there has been significant effort devoted to developing anticancer agents targeted against Cdc25 phosphatases.

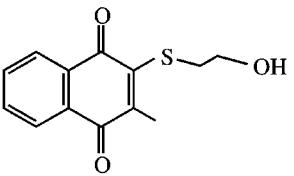
Many of the reported Cdc25 inhibitors are natural products and their derivatives (Figure 5). For example, sulfircin (Compound 15), a marine natural product isolated from a deep water sponge of the genus *Ircinia*, exhibited an IC_{50} of 7.8 μM against Cdc25A (95). However, biological testing of sulfircin and its synthetic analogs against a panel of PTPs indicated limited selectivity. Another natural product, dysidiolide (Compound 16), which was isolated from the marine sponge *Dysidea etheria*, was reported to inhibit Cdc25A with an IC_{50} of 9.4 μM (96). Dysidiolide was also found to inhibit growth of the A-549 human lung carcinoma and P388 murine leukemia cell lines with IC_{50} values of 4.7 and 1.5 μM, respectively. A synthetic vitamin K analogue, Compound 17, caused a time-dependent, irreversible inactivation of Cdc25 phosphatases by sulfhydryl arylation of the catalytic cysteine and induced cell-cycle arrest and inhibited tumor cell growth (97). Based on a common pharmacophore derived from protein Ser/Thr phosphatase inhibitors–calyculin, microcystins, and okadaic acid–a targeted library was designed and synthesized, which led to the identification of a novel Cdc25B inhibitor, FY21-$\alpha\alpha$09 (Compound 18) (98). Compound 18 displayed moderate selectivity against Cdc25B with a K_i of 1.6 μM and blocked the proliferation of human breast cancer cells. Most recently, several steroidal derived Cdc25A



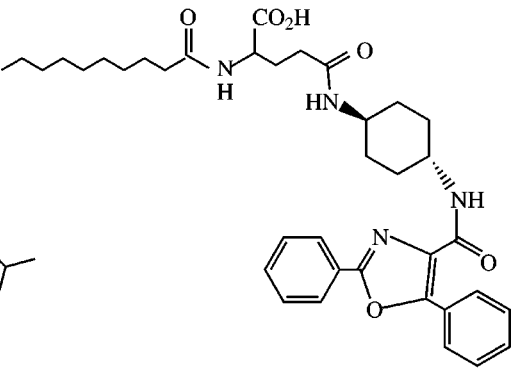
15, Sulfircin, IC_{50} =7.8 μM
ref. 95



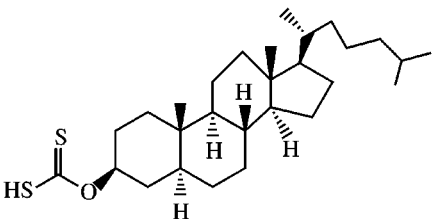
16, Dysidiolide, IC_{50} =9.4 μM
ref. 96



17, IC_{50} = 3.8 μM, ref. 97



18, FY21-αα09, K_i = 1.6 μM, ref. 98



19, IC_{50} = 1.1 μM, ref. 99

Figure 5 Structures of small-molecule Cdc25 inhibitors.

inhibitors were shown to have antiproliferative activities in the NCI60-human tumor cell line screen (99). Some of the compounds (e.g., Compound 19) exhibited potent selectivity for Cdc25A ($IC_{50} = 1.1\ \mu M$) over CD45 ($IC_{50} > 100\ \mu M$), a tyrosine-specific PTP.

CONCLUDING REMARKS

The importance of PTPs in the control of cellular signaling is well established. Furthermore, there are compelling reasons to believe that PTP inhibitors may serve as novel medicinal agents for the treatment of various diseases. However, because

PTPs can both enhance and antagonize PTK signaling, it will be critical to elucidate the physiological context in which PTPs function. One of the major challenges of current research is to be able to establish the exact functional role for each individual PTP both in normal cellular physiology and in pathogenic conditions. One obvious strategy to achieve this goal is to apply gene knockout and RNA interference technologies. Another strategy will be the use of small-cell permeant compounds that are specific inhibitors of particular PTPs. Despite the difficulties in obtaining such compounds, there are now several relatively specific inhibitors for PTP1B. Further understanding of the specific functional roles of PTPs in cellular signaling also requires detailed investigation of PTP-catalyzed substrate dephosphorylation. Understanding the molecular basis for protein dephosphorylation will promote development of new experimental approaches (such as the creation of PTPs with altered catalytic and regulatory properties and the design and development of specific PTP inhibitors) that will reveal mechanisms by which these enzymes control critical cell functions. Detailed structural and mechanistic information on PTPs will continue to be of considerable importance and will provide a solid basis for inhibitor design. As already demonstrated for PTKs, it should be possible to design and develop specific small-molecule PTP inhibitors that would enable the pharmacological modulation of selected signaling pathways for the treatment of various diseases.

ACKNOWLEDGMENTS

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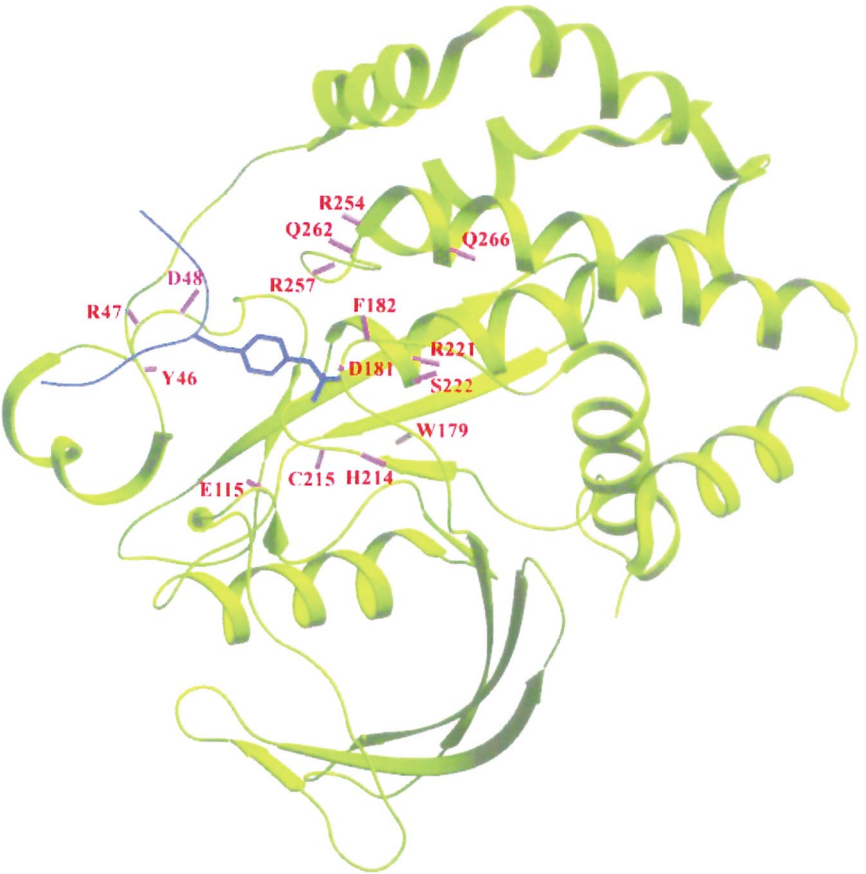

Figure 1 Structure of PTP1B (*yellow-green*) bound with the consensus peptide substrate ELEFpYMDYE (*blue*) (38). Purple sticks representing the main chain carbonyl groups indicate locations of residues that have been examined by mutagenesis.