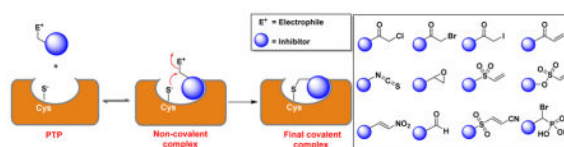# Covalent inhibition of protein tyrosine phosphatases

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Abstract

Protein tyrosine phosphatases (PTPs) are a large family of 107 signaling enzymes that catalyze the hydrolytic removal of phosphate groups from tyrosine residues in a target protein. The phosphorylation status of tyrosine residues on proteins serve as a ubiquitous mechanism for cellular signal transduction. Aberrant function of PTPs can lead to many human diseases, such as diabetes, obesity, cancer, and autoimmune diseases. As the number of disease relevant PTPs increases, there is urgency in developing highly potent inhibitors that are selective towards specific PTPs. Most current efforts have been devoted to the development of active site-directed and reversible inhibitors for PTPs. This review summarizes recent progress made in the field of covalent inhibitors to target PTPs. Here, we discuss the *in vivo* and *in vitro* inactivation of various PTPs by small molecule-containing electrophiles, such as Michael acceptors, α-halo ketones, epoxides, and isothiocyanates, etc. as well as oxidizing agents. We also suggest potential strategies to transform these electrophiles into isozyme selective covalent PTP inhibitors.

Graphical Abstract

Chemical strategies for covalent inhibition of protein tyrosine phosphatases.

Introduction

Tyrosine phosphorylation of proteins plays an important role in a variety of cellular processes such as proliferation, migration, and apoptosis. The tyrosine phosphorylation status of a target protein is controlled by the balanced and opposing action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).[1] PTKs transfer the γ-phosphoryl group (PO_3^{2-}) from ATP to the tyrosine residue on a target protein, whereas PTPs remove the phosphate group to generate the dephosphorylated target protein and an inorganic phosphate.[1] Dysregulation of both PTKs and PTPs are associated with pathological processes such as cancer, diabetes, obesity, and autoimmune disorders.[2] PTKs

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are a family of 90 enzymes encoded by the human genome.[3–5] Currently, over 20 small molecule inhibitors targeting PTKs have already been approved by FDA to treat various cancers, and many other inhibitors are currently under clinical development.[2,6] Of the ~107 PTPs encoded by the human genome, at least 37 have been associated with human cancer and approximately an equal number of PTPs are involved in oncogenic and tumor suppressor activities.[7,8] However, no single drug targeting PTPs has yet been commercialized. PTPs are extremely difficult targets for drug discovery, as over two decades of intense efforts by academia and the pharmaceutical industry generated only a small number of potent and selective PTP inhibitors with robust *in vivo* activity.[9–11] The vast majority of PTP inhibitors reported in the literature are phosphotyrosine (pTyr) mimetics that occupy an active-site and extend into the surrounding region to gain selectivity. Several highly potent two-site binding small molecules have been reported as inhibitors of PTPs.[12–16] Recently, allosteric inhibition strategies have generated promising small molecule inhibitors that are potent and selective towards some of the disease relevant PTPs. Chen *et al.* discovered a highly potent (IC$_{50}$ = 0.071 μM), selective and orally bioavailable inhibitor SHP099 for SHP2, the first oncogenic phosphatase in the PTP family.[17–19] SHP2 in its basal state exists in an autoinhibited closed conformation due to an interaction between its N-terminal Src homology 2 (SH2) domain and the PTP domain. Binding of phosphotyrosine (pTyr) ligands to SH2 domains disrupts the autoinhibitory interaction that leads to activation of the phosphatase.[20] SHP099 inhibits SHP2 by stabilizing it in an auto-inhibited conformation. Trodusquemine (MSI-1436) was characterized as a selective and non-competitive inhibitor of PTP1B,[21] a negative regulator of insulin and leptin signaling.[22–25] MSI-1436 binds to the C-terminal segment of PTP1B that allosterically locks PTP1B in an inactive conformation. More recently, strategies to selectively target oxidized PTPs were also reported. By utilizing a double mutant form of PTP1B (CASA) as an antigen that mimics the active site Cys oxidized form of PTP1B, a single chain of variable fragments (scFvs) was developed to recognize and stabilize an oxidized PTP1B in the inactive state inside a cell.[26] Protein oligomerization is also known to play an important regulatory role in the activity of some PTPs. Compound 43 was discovered by *in silico* library screening as an oligomerization inhibitor of phosphatase of regenerating liver 1 (PRL1).[27] Compound 43 inhibits PRL1 trimerization without affecting catalytic activity, and also blocks PRL1-induced cell proliferation and migration through reduced ERK1/2 and Akt activity.

The strategy of using an inhibitor with a reactive group (often an electrophile) to covalently modify a target protein is gaining interest among the research community. Covalent inhibitors have several advantages, such as low dose requirement for achievable high potency, prolonged duration of action (due to covalent bond formation), and resistance to mutations.[28,29] However, off-target interactions that are also long lasting can have toxic side effects. For instance, covalent modification of cytochrome P450 by electrophilic reagents leads to severe side effects.[30] Though the covalent inhibition strategy has, in general, been avoided by the pharmaceutical industry due to a widely perceived increase in toxicity from covalent drugs, there are many examples of blockbuster drugs, such as aspirin, penicillin, omeprazole, and clopidogrel, that exert their pharmacological effects through covalent modification of their respective target proteins. Indeed, clopidogrel (to prevent cardiovascular diseases), lansoprazole, and esomeprazole (proton pump inhibitors)

constituted three of the ten top-selling drugs in the United States in 2009.[31] Currently, there are approximately 42 approved covalent drugs to treat diseases such as infections, cancer, and cardiovascular.[28,32] In fact, there is no correlation between thiol conjugate formation and the *in vivo* toxicity of approved covalent drugs.[33] Recent successes of covalent kinase inhibitors has prompted a renewed interest in developing covalent inhibitors for drug discovery.[34] Given the requirement of the active site Cys for PTP catalysis, PTPs represent prime targets for covalent inhibition.[35] Some aspects of covalent inhibition and activity based probes targeting PTPs have been covered by two recent mini-reviews.[36,37] Here, we provide a comprehensive summary and an update on recent progress on efforts to develop covalent inhibitors (both reversible and irreversible) to target PTPs.

Classification of PTPs

PTPs are Cys dependent phosphatases with a conserved active site motif $C(X)_5R$ that can be broadly divided into four groups based on their structure and substrate specificity.[7] Group I consist of classical PTPs (38 members) that are specific for phosphotyrosine (pTyr). The classical PTPs are strictly tyrosine-specific and are further subdivided into two categories based on their subcellular localization: intracellular PTPs (e.g., PTP1B, SHP, HePTP, STEP, etc.) and receptor-like PTPs (e.g., CD45, LAR, PTPα, PTPβ, PTPσ, etc.). Group II contains dual-specific phosphatases (DSPs, 61 members) that can hydrolyze phosphate from tyrosine, serine/threonine, phosphatidylinositol and glycogen. Thus, the DSPs (VH1-like enzymes) are the most diverse group in terms of substrate specificity and can be divided into several subgroups that share less sequence identity with each other. The DSP family of PTPs contains highly specialized types of phosphatases; members of this family include phosphatases of regenerating liver (PRLs), mitogen-activated protein kinase phosphatases (MKPs), myotubularins, phosphatase and tensin homologue (PTEN) type phosphatases, atypical DSPs, CDC14s, and slingshots. Group III consists only one member, the low-molecular-weight phosphotyrosine phosphatase (LMW-PTP). In humans, this class is represented by an 18 kDa tyrosine-specific low molecular weight phosphatase. The activity of LMW-PTP is regulated by reversible oxidation of cysteine residues and the tyrosine phosphorylation status.[38] Group IV PTPs are represented by three phosphatases: CDC25A, CDC25B, and CDC25C (CDC refers to cell division cycle). These three cell cycle regulators dephosphorylate cyclin-dependent kinases (CDKs) at their inhibitory N-terminal Thr/Tyr motifs.[39] This dephosphorylation leads to the activation of these CDKs to drive cell cycle progression.[40] Overexpression of CDC25B has been correlated to the malignancy of tumors.[39,41] All PTPs are cysteine-based enzymes that catalyze a dephosphorylation reaction in which an active-site cysteine thiolate (RS⁻) acts as a nucleophile and attacks the phosphorus center of the substrate phosphoryl group.[35]

Mechanism of PTP catalysis

All PTPs share a common catalytic domain of approximately 250 amino acids made of a central parallel β–sheet with flanking α-helices containing a β-loop-α-loop that encompasses the PTP signature motif (I/V)HCXXGXXR(S/T), also called the catalytic loop or P-loop.[42] This loop consists of 11 amino acid residues, of which cysteine and arginine are critical for catalytic activity. Key residues essential for PTP catalysis are depicted in the

structure of the catalytic domain of PTP1B (Fig. 1). The thiolate group of the invariant active site cysteine (Cys215 in PTP1B) functions as the attacking nucleophile to initiate phosphate monoester hydrolysis. The active site arginine (Arg221 in PTP1B) is a highly conserved residue among PTPs and is essential for phosphotyrosine substrate binding; it also stabilizes the transition state by forming a pair of hydrogen bonds with the phosphate oxygen atoms.[43,44] The WPD loop (tryptophan, proline, and aspartic acid) is also a conserved loop in PTPs and is a flexible surface loop. Crystallographic studies revealed that the WPD loop can take either an open or closed conformation.[44,45] A unique aspartic acid located in the WPD loop (Asp181 in PTP1B) acts a general acid/base catalyst in the dephosphorylation reaction. The pTyr loop contains a tyrosine residue (Tyr46 in PTP1B) that defines the depth of the active site cleft and contributes to the specificity of pTyr-containing substrates.[46]

PTPs exert their catalytic activity by using a nucleophilic cysteine located at the bottom of the active site. This active-site cysteine residue predominantly exists as a thiolate (RS⁻) at physiological pH, due to its unusually low pK_a of ~4.5.[47,48] The dianionic phosphotyrosine (pTyr) residue of the substrate binds to the positively charged active site of PTP by an array of hydrogen bonds with the guanidine side chain of arginine in the P-loop and backbone amide residues. Nucleophilic attack of cysteine thiolate onto the phosphorus atom of the substrate phosphoryl group leads to formation of a phospho-cysteine intermediate (Fig. 2).[49–51] In this step, a conserved aspartic acid residue in the WPD loop acts as a general acid that protonates the tyrosyl-leaving group. [52,53] In the next step, an activated water molecule attacks at the phospho-cysteine intermediate (catalyzed by the same conserved Asp in the WPD loop as a general base); this leads to release of inorganic phosphate and regenerates the cysteine thiolate.[46] This step is mediated by the conserved glutamine (Gln262 in PTP1B) present in the Q-loop, which coordinates with a water molecule by hydrogen bonding interactions.[54]

Role of PTPs in diseases

PTPs were once considered as housekeeping enzymes that function with no or little substrate specificity.[55] However, over the past two decades research has demonstrated the essential roles of PTPs in many human diseases. PTP1B was the first PTP purified to homogeneity, and experiments by Elchebly *et al.* and Klaman *et al.* on PTP1B knockout mice revealed its role in insulin and leptin signaling pathways.[24,25,56,57] The PTP1B knockout mice displayed higher insulin sensitivity, lower glucose levels, and resistance to obesity with a high fat diet. Moreover, increased PTP1B activity and expression in adipose tissue and skeletal muscle of obese insulin-resistant human subjects were also reported.[58,59] Other PTPs, including TC-PTP, LMW-PTP, and PTP-MEG2, have also been implicated as negative regulators of insulin and/or leptin signaling.[60–62] Consequently, there is great interest in developing inhibitors to target these PTPs as apeutic agents for type 2 diabetes and obesity. More recently, Krishnan *et al.* showed that inhibition of PTP1B may reverse the symptoms of Rett Syndrome (RTT), a neurological disorder similar to that of Huntington's disease and cystic fibrosis.[63] Src-homology 2 domain-containing phosphatase 2 (SHP2), encoded by the *Ptpn11* gene, promotes signaling downstream from cytokine and growth factor receptors and upstream of Ras. Germline mutations in SHP2 were linked to Noonan and LEOPARD syndromes. Activating mutations of SHP2 have been found to be associated with multiple cancers, such

as lung cancer, breast cancer, colon cancer, prostate cancer, leukemia, neuroblastoma, glioblastoma, and melanoma.[16,64,65]

CDC25 family phosphatases play critical roles in eukaryotic cell cycle control. They promote activation of cyclin-dependent kinases (CDKs) by removing the inhibitory phosphate(s) in CDKs.[66] Overexpression of CDC25 phosphatases were found in a number of human cancers, including head, neck, breast, colon, lung, gastric, pancreatic, and neuroblastoma.[67] CDC25 overexpression was observed at all stages of tumorigenesis, indicating their roles throughout tumor development. STriatal-enriched protein tyrosine phosphatase (STEP) is a neuron-specific phosphatase with two alternative spliced isoforms (STEP46 and STEP61). STEP regulates the activity of mitogen-activated protein kinase (MAPK) family members ERK1/2 and p38, the tyrosine kinases Fyn, and Pyk2, and the glutamate receptor GluN2B subunit of NMDARs.[68] Genetic reduction of STEP improves cognition in Alzheimer's disease (AD) mouse models, thus identifying STEP as a promising therapeutic target for the treatment of AD.[69] The phosphatases of regenerating liver (PRLs) are a subfamily of three dual specificity phosphatases. All three PRLs, PRL1/2/3 promote cell proliferation, migration, invasion, tumor growth, and metastasis.[70] PRL3 is the most studied of the three PRLs and it is overexpressed in liver metastatic tissue from colon cancer, but not in normal colon tissue. PRL-3 was also found to be overexpressed in many other human cancer types, such as melanomas, gastric, ovarian, breast, acute myeloid leukemia, and Hodgkin's lymphoma.[70] CD45 (lymphocyte common antigen), a class I receptor-like PTP, promotes antigen receptor signaling in lymphocytes.[71] CD45 and CD45-associated molecules play essential roles in T cell and B cell receptor signaling. Due to its important role in immune signaling pathways, CD45 has been linked to many autoimmune diseases.[2] For instance, a single-nucleotide polymorphism (C77G) in exon 4 of CD45 is associated with increased incidence of multiple sclerosis, HIV, systemic sclerosis, and autoimmune hepatitis.[72] Lymphoid tyrosine phosphatase (LYP), encoded by the *Ptpn22* gene, is associated with a wide spectrum of autoimmune disorders such as, rheumatoid arthritis, type 1 diabetes, systemic lupus erythematosus, and Graves' disease.[73] A hallmark of cancer cells is their ability to evade apoptosis. RNA interference (RNAi) screens to identify anti-apoptosis genes in HeLa cells revealed 28 human PTP genes as positive regulators of cell survival, and 4 PTPs as cell death phosphatases.[74] This study demonstrated the regulatory role of PTP activity in maintaining cellular homeostasis. As described above, dysregulation of PTP activity has been linked to many human diseases, such as cancer, diabetes/obesity, autoimmune disorders, neurological diseases, and infectious diseases. Therefore, a majority of the members of PTP family are molecular targets for drug discovery.

Kinetic parameters for covalent inhibitors

Covalent inhibitors work by forming a covalent bond with a reactive functional group in the target enzyme. Consequently, the kinetics for enzyme inactivation by a covalent inhibitor is not an equilibrium process as with reversible inhibition. The potency of a reversible inhibitor can be measured by its IC_{50} value.[75] The IC_{50} is the concentration of the inhibitor at which 50% of the target enzyme is inhibited. The Cheng-Prusoff equation can be used to convert IC_{50} into an inhibition constant K_i.[76] Unlike reversible inhibitors, where inhibition is only concentration dependent, covalent inhibitors show both time and concentration dependent

target inhibition (a covalent bond formation is a time-dependent event). If sufficient time is available, covalent inhibitors can completely neutralize the target rather than go to equilibrium (Fig. 3). As a result, potency and selectivity of a covalent inhibitor cannot be determined by IC_{50} values; instead, the rate of inactivation of the target (k_{inact}) and inhibition constant (K_I) are preferred. Most often, the bimolecular rate constant (k_{inact}/K_I), is chosen to rank the potency of covalent inhibitors.[28,75,77] Compared to IC_{50} determination, obtaining k_{inact}/K_I for covalent inhibitors is more laborious and involves two steps. In a binding assay, the extent of inhibition is measured over time at different inhibitor concentrations. The observed rate constant (k_{obs}) for each concentration is obtained from the slope of a semi-logarithmic plot of inhibition *vs* time. The k_{obs} values are re-plotted against inhibitor concentration and fitted to a hyperbolic equation (Equation 1) to determine K_I and k_{inact} values.[78] in cases where it is difficult to derive both K_i and k_{inact} (low concentrations of inhibitors), one can obtain the bimolecular rate constant via a k_{obs} versus [inhibitor] plot.

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]} \qquad \text{Equation 1}$$

Covalent PTP inhibitors

Many compounds have been reported that are capable of inactivating the PTPs. We classified these PTP covalent inhibitors into eight categories based on the reacting (warhead) groups present in the molecule for covalent modification of active site residues of the PTPs.

1. Oxidizing agents

Due to the highly nucleophilic nature of the catalytic cysteine residue in PTPs, the active site thiol group is prone to oxidation that causes either transient or permanent loss of phosphatase activity in cells. Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) produced during a variety of signaling events, are capable of modulating PTP activity by oxidative inactivation.[47] This inactivation involves conversion of the catalytic cysteine residue to a sulfenic acid, a sulfenyl amide, or a disulfide (Fig. 4).[47,79–81] Oxidation of the active site thiol group to sulfenic acid (SOH) or sulfenyl amide is a reversible event, but its over-oxidation to sulfinic acid (SO_2H) or sulfonic acid (SO_3H) causes permanent loss of PTP catalytic activity. In the case of PTP1B and PTPα, the sulfenic acid quickly loses a water molecule to form a relatively stable cyclic sulfenyl amide moiety (Scheme 1).[82–84] Reaction of this sulfenyl amide with cellular thiols regenerates the enzyme activity *via* formation of a mixed disulfide.[85] On the other hand, oxidation of active site thiol of CDC25C,[86] PRL1,[87] PTEN[80] and LMW-PTP[88] results in the formation of a disulfide bond with a neighboring cysteine residue. This redox chemistry of PTPs by ROS serves as a regulatory mechanism under certain physiological conditions. Increased levels of cellular oxidants, in response to growth factors, triggers the tyrosine phosphorylation dependent signaling by transient inactivation of members of the PTP family.[89] For instance, during insulin signaling events, a burst of H_2O_2 causes inactivation of PTP1B *via* oxidation of the active site cysteine thiolate (Cys 215) to a sulfenyl amide moiety. Reaction with

cellular thiols then slowly regenerates the active enzyme that terminates cellular responses to insulin.[82]

In vitro analysis of hydrogen peroxide-mediated inactivation of PTPs revealed that hydrogen peroxide is a sluggish inactivator of PTPs with rate constants of 10–20 $M^{-1} s^{-1}$. LaButii *et al.* reported peroxymonophosphate ($^{2-}O_3POOH$) as an exceptional inactivator of PTP1B with $K_I = 6.6 \times 10^{-7}$ M and $k_{inact} = 0.043 s^{-1}$.[90] As a result, peroxymonophosphate ($k_{inact}/K_I = 65,553 M^{-1} s^{-1}$) is approximately 7,000 times more potent than hydrogen peroxide, an endogenous regulator of PTP1B.[91] Like hydrogen peroxide, peroxymonophosphate is also an active-site directed and thiol reversible inactivator of PTP1B. In a recent study, Meng & Zhang showed that hydroxyl radical could also be a viable oxidizing agent for the PTP active site under physiological conditions.[92] Importantly, second-order rate constants of hydroxyl radical-mediated inactivation of PTPs are 2–3 orders of magnitude higher than those mediated by hydrogen peroxide under identical experimental conditions.

In an attempt to discover organic compounds that could modulate PTP1B activity by redox mechanism, Bhattacharya *et al.* screened a set of organic hydroperoxides (1–6) against PTP1B (Fig. 5).[93] Peracetic acid 1 showed time-dependent inactivation of PTP1B, which is consistent with covalent modification of PTP1B. The site of covalent modification was confirmed by competition experiments with phosphate ion. The presence of phosphate ion caused approximately 40% decrease in the inactivation power of 1, indicating the active-site directed modification of the enzyme. Further analysis revealed the reversible nature of the inactivation, where 73% of initial activity was restored when 1 modified PTP1B was treated with low molecular weight thiol. Compounds 5 and 6 showed superior inactivation kinetics compared to 1, which may be attributed to the non-covalent interaction of aromatic rings with pTyr binding residues such as Tyr46 and Phe182 prior to covalent modification. *tert*-butyl hydroperoxide 2 and cumene hydroperoxide 3 showed no inactivation of PTP1B.[90] Steric bulk of the tertiary group could be preventing these two compounds from penetrating the deep active-site pocket of PTP1B.

Nitric oxide (NO) produced in biological systems is known to play important functional roles in numerous processes in the cardiovascular, immune, and nervous systems.[94] Post-translational modifications through S-nitrosylation of protein thiols by nitric oxide is well documented.[95,96] Caselli *et al.* conducted *in vivo* and *in vitro* experiments to investigate the role of nitric oxide on LMW-PTP and *Yersinia* PTP. In the case of LMW-PTP, mass spectrometric analysis of tryptic fragments confirmed oxidation of Cys12 and Cys17 to form a disulfide bond.[97] *In vivo* inhibition of phosphatases by NO was also observed in RAW 264.7 murine macrophages. These studies confirm the ability of NO to inactivate PTPs and suggests a possible role of nitric oxide in the redox regulation of cellular processes involving PTPs.[98]

Takakura *et al.* investigated the effects of biological oxidants such as peroxynitrite, and *S*-nitrosoglutathione (GSNO) on the activity of three human PTPs, CD45, PTP1B and LAR.[99] All these PTPs were irreversibly inactivated by peroxynitrite in less than 1 s with IC_{50} values of less than 0.9 μM. The bimolecular reaction rates for peroxynitrite mediated

inactivation of PTP1B, LAR, and CD45, were 2.2×10^7, 2.3×10^7, and 2.0×10^8 M^{-1}s^{-1}, respectively. These high reaction rates suggest that PTPs could be inactivated by peroxynitrite even in the presence of thiol reagents. Incubation of peroxynitrite-inactivated LAR with 20 mM DTT recovered only about 10% of the original activity; similar results were observed with PTP1B and CD45, indicating the oxidation of active site thiol to sulfinic acid (OSO$_2$H) or even to sulfonic acid (OSO$_3$H) which cannot be reduced by biological thiols such as DTT.[47] Conversely, incubation of LAR with 1 mM of GSNO for 30 min showed 50% of inhibition and the inhibition was completely reversed by DTT and β-mercaptoethanol. These results demonstrate that any PTP with sequence CX$_5$R in the active-site could be oxidized by peroxynitrite in cells, causing an increase in tyrosine phosphorylation and thereby impacting tyrosine phosphorylation dependent signaling events.

In an another study, Ming and co-workers demonstrated low-molecular weight *S*-nitrosothiols such as GSNO, *S*-nitroso-captopril, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), and S-nitrosylated human serum albumin as time and concentration dependent inhibitors of mammalian PTP1B and *Yersinia* PTP (YopH) (Fig. 6).[100] These four S-nitrosothiols exhibited k_{inact}/K_I values ranging from 37 to 113 M^{-1} min^{-1} and 66 to 613 M^{-1} min^{-1} against PTP1B and YopH, respectively (Table 1). Moreover, both single-S-nitrosylated and poly-S-nitrosylated human serum albumin showed second order rate constants of 472 and 1,188 M^{-1} min^{-1}, against YopH, respectively. This indicates a possible role of *in vivo* inactivation with a variety of cysteine-dependent enzymes by S-nitrosylated albumin. In an another instance, Barrios research group reported oxidative inactivation of LYP with sodium nitroprusside (SNP) (Fig. 6) and a novel peptide-based nitrosating reagent, Ac-ARLIEDNE(HcyNO)TAREG-NH2 (where Hcy-NO = S-nitrosohomocysteine).[101] The SNP inactivated LYP with an equilibrium binding constant (K_I) of 27.4 μM, an inactivation rate constant (k_{inact}) of 0.383 min^{-1}, and mixed-type inactivation kinetics. This peptide showed competitive LYP inactivation kinetics with a K_I of 7.00 μM and k_{inact} of 0.0472 min^{-1}. Inactivation of LYP by SNP resulted through the formation of a LYP-NO adduct, whereas inactivation by the peptide did not result in formation of an LYP-NO adduct. Based on these observations, it was proposed that general NO donors nitrosate any free cysteine residue while the active-site directed peptide selectively nitrosates the active site cysteine, resulting in formation of a disulfide bond between the active site cysteine residue and a neighboring cysteine.

2. Phosphates and phosphonates

A general strategy to develop inhibitors for PTPs is to design a nonhydrolyzable phosphonate moiety that mimics the phosphate group of pTyr. However, replacement of the phenolic oxygen atom with a non-polar methylene group may decrease affinity for the active site and also reduce water solubility.[102] To address these issues, medicinal chemists replaced the hydrogens on the α-carbon with halogen atoms, to make the phosphonate an effective electronic isostere of the phosphate group. Chen *et al.* synthesized phosphonodifluoromethyl phenylalanine (F$_2$Pmp) as a nonhydrolyzable pTyr mimetic. Presence of the difluoromethyl group increased the affinity of a F$_2$Pmp-containing peptide over a Pmp-containing counterpart by 1,000-fold towards PTP1B. This increased affinity may be due to hydrogen

bonding interactions of the two fluorine atoms, similar to the phosphate ester oxygen in pTyr.[11,103,104]

In 1996, Taylor *et al.* examined α-halobenzylphosphonates as possible inactivators of PTPs. Compounds 7, 8, and 9 (Fig. 7) were screened for inhibition against Yop51 PTP from *Yersinia enterocolitica.* A direct comparison of these compounds revealed that bromide 9 is a superior inactivator compared to chloride 8 and fluoride 7, which showed modest and no inactivation, respectively. Decrease in the inactivation rate of bromide 9 in the presence of *p*-nitrophenyl phosphate (substrate) or arsenate (known competitive inhibitor of the enzyme) confirmed the active site directed inactivation (Fig. 7).[105] Later, Tulsi *et al.* prepared L-phosphonobromomethylphenylalanine (BrPmp) derivatives 10 and 11 as irreversible inhibitors of PTP CD45. The Kitz–Wilson analysis estimated the rate of irreversible inhibition (k_{inact}) of CD45 as 0.05 min^{-1} for both 10 and 11, and K_I values of 40 μM and 16 μM, respectively. These results indicate that tripeptide 11 was more potent than BrPmp alone, suggesting additional interactions of the peptide side chains to improve enzyme binding.[106]

In 2004, Zhang and co-workers utilized the α-bromobenzylphosphonate (α-BBP) moiety to design activity-based probes to facilitate global analysis of PTPs.[107] They synthesized probes 12 and 13, where α-BBP acts as a PTP-specific trapping device and a linker with a biotin tag for capture and visualization of PTP activity. Two possible mechanisms were proposed for the action of these probes against PTPs (Fig. 8). In one of the mechanisms, the active site cysteine thiolate attacks the phosphorus atom of the probes to yield a S-P covalent bond (Fig. 8A). In another mechanism, the general acid/base Asp residue (note the Cys is not in a position to make this attack based on its structure) attacks the carbon bearing the bromine atom in an S_N2 fashion to form a stable ester linkage (Fig. 8B). Site-directed mutagenesis experiments indicated that a covalent adduct was formed between α-BBP and PTP involving the active site Cys residue, consistent with mechanism A in figure 8. These α-BBP-based agents showed broad reactivity against a set of PTPs in an active site-directed, irreversible fashion. Interestingly, these probes are inert toward serine/threonine, acid or alkaline phosphatases, and serine or cysteine proteases, validating their utility as broad spectrum mechanism-based probes. The selectivity of these agents may be due to the structural resemblance between α-BBP and phosphotyrosine and the juxtaposition of the electrophilic group to the active-site nucleophile. Kumar *et al.* incorporated rhodamine tags in place of the biotin into α-BBP derivatives to detect PTPs in various cancer cell lines.[108] Rhodamine probes showed reactivity and selectivity profiles similar to the biotin probes against PTPs and demonstrated 1,000 fold more sensitivity than the biotin probes.

4-Difluoromethylphenyl bis(cyclohexylammonium) phosphate 14 (Fig. 7), was reported as a mechanism-based inactivtor of human prostatic acid phosphatase and SHP protein tyrosine phosphatase.[109] This 4-difluoromethylphenyl phosphate (DFPP) derivative is inert itself, but upon hydrolysis of the phosphate group leads to the spontaneous elimination of a fluoride ion, which generates a highly reactive electrophilic quinone methide group. This quinone methide can be attacked by nucleophiles in an active-site to give stable Michael addition products (Fig. 9). The inactivation of human prostatic acid phosphatase and SHP by 14 followed pseudo first order kinetics. The inactivation rate constants for human prostatic acid

phosphatase reported as $k_{inact} = 0.15$ min^{-1} and $K_I = 1$ mM. This quinone methide approach has been used by several research groups to design PTP activity based probes,[36,110,111] although it should be noted that 4-fluromethylaryl phosphate is not specific for PTPs, as it also reacts with other phosphatases such as prostatic acid phosphatase and Ser/Thr phosphatase calcineurin.[109,112,113]

Cyclic phosphate ester 2-Methoxy-4H-1,3,2-benzodioxaphosphor-2-one, also known as salioxon, is a covalent inhibitor of esterases (Fig. 7). Due to its structural resemblance with phenyl phosphate, which is a substrate of PTPs, it was expected to inactivate PTPs. Salioxon showed irreversible inhibition of PTP1B, YPTP1, and SHP-1, although the compound is a rather weak inactivator of these PTPs.[114] Unfortunately, no kinetic or mechanistic studies of PTP inhibition by salioxon were reported. Salioxon is an interesting example of a nonhydrolyzable pTyr mimetic, and may be modified at phenyl ring and/or cyclic phosphate group to generate more potent and selective inactivators for PTPs.

3. α-halo carbonyls

Alpha halo carbonyl compounds present an electrophilic center at the α-carbon atom that can be attacked by nucleophilic residues in proteins.[48,115] Pot & Dixon first reported iodoacetate as an inactivator of the cytoplasmic PTP domain of LAR, a receptor-like PTP.[115] Inactivation of LAR by iodoacetate was time dependent and the loss of catalytic activity followed pseudo-first order kinetics. Surprisingly, the structurally similar iodoacetamide (Fig. 10) showed no inhibition against LAR.[116] Zhang and Dixon (1993) investigated iodoacetate *vs* iodoacetmide as inactivators of the *Yersinia* PTP and rationalized the difference in reactivity between the two compounds.[48] According to their findings, iodoacetate exhibits selective and stoichiometric labeling of active site cysteine (Cys403) of *Yersinia* PTP. In addition, iodoacetate showed 940-fold more reactivity toward *Yersinia* PTP compared to iodoacetamide. This differential reactivity correlates with the positively charged nature of the residues within the active-site of the enzyme.

In 1999, Pei and co-workers reported α-haloacetophenone derivatives as photocleavable covalent inhibitors of PTPs.[117,118] The authors envisioned the α-haloacetophenone moiety as a pTyr mimetic, where the electron-rich halogen atom could mimic the phosphate group, and the phenyl ring could provide hydrophobic interactions with the active site of a PTP similar to that of the phenyl ring in a natural substrate. Such a binding would place the highly electrophilic α-carbon of the α-haloacetophenone next to the catalytic cysteine thiolate of the PTP for an S_N2 reaction. This would result in loss of phosphatase activity *via* the formation of a stable thioether linkage. In this study, compounds 15, 16, 17, and 18 (Fig. 10) were assayed in an inactivation experiment against PTP1B, SHP1, the catalytic domain of SHP1 (SHP1   SH2), and LAR. All four compounds showed time and concentration dependent inactivation of the PTPs tested. Inhibitor 15 showed the highest affinity, with k_{inact} values of 0.57 and 0.40 min^{-1}, and K_I values of 42 and 43 μM for PTP1B and SHP1(SH2), respectively. The α-bromo derivative 17 is 13-fold more potent than its α-chloro counterpart 18; this may be due to either the smaller size of the chlorine atom in α-chloroacetyl group making it a less effective pTyr mimetic compared to the large bromine atom or the presence of a better leaving group Br in 17. MALDI mass spectrometric analysis

of the trypsin digested fragments of 17, inactivated SHP1, and kinetic analysis of C453S mutant experiments were consistent with the covalent attachment of 17 to the active-site Cys 435 of SHP1. Western-blot analysis of B cell phosphoproteins showed increased pTyr levels in 17-treated B cells. To improve potency, selectivity, and membrane permeability of the α-haloacetophenone core, the same research group undertook a SAR study.[118] They prepared over twenty bromo- and chloro- acetophenone derivatives and tested their efficacy against SHP1 and PTP1B. Compounds with altered electronic properties of the phenyl ring showed no significant improvement in potency against PTPs. However, compounds 19 and 20, bearing a tripeptide (Gly-Glu-Glu) group attached *via* a peptide bond, showed highest potency against SHP1 and PTP1B. Analogue 19 exhibited a K_I of 2.8 mM, k_{inact} of 1.2 min^{-1}, and specificity constant (k_{inact}/K_I) of 4.3×10^5 M^{-1} min^{-1} against PTP1B. It also showed 25-fold improved potency against SHP1 compared to the parent analogue 14. While inhibitor 20 has a k_{inact}/K_I value of 3.6×10^4 M^{-1} min^{-1} and 666 M^{-1} min^{-1} against PTP1B and SHP1, respectively, showing a 54-fold selectivity toward PTP1B. The inactivation by these agents quickly reversed upon irradiation of UV light at 350 nm (Fig. 11). For example, approximately 80% of original activity was recovered by the irradiation of 17 modified SHP-1 for ~15 min (Fig. 11). These molecules could provide a novel class of photo-reversible PTP inhibitory agents for controlling cellular signaling processes.

4. α,β-unsaturated compounds

The Michael addition is the most commonly used reaction to achieve covalent modification of proteins. Typically, functional groups that undergo this addition reaction include α,β-unsaturated carbonyls, vinyl sulfones, vinyl sulfonates, quinones, alkynyl amides, propargylic acid derivatives, and cyanoacrylates.[119,120] The Michael addition of cysteine thiol to these functional groups is a reliable way to selectively modify cysteine residues in target proteins.[121]. Seiner *et al.* reported acrolein as a potent time-dependent inactivator of PTP1B (k_{inact} = 0.02 s^{-1} and K_I = 2.3×10^{-4} M).[122] The authors determined percentage enzyme activity that remained for a series of structurally related aldehydes against PTP1B (Fig. 12). When incubated with 225 nM of PTP1B, acrolein, crotonaldehyde, 3-methyl-2-butenal, glyoxal, and propanal (all 500 μM, for 10 min) showed 4%, 83%, 93%, 95%, and 93% of remained enzyme activities, respectively. This suggests that the double bond in acrolein is essential for the inactivation of PTP1B. In addition, steric bulk at the alkene terminus weakens the inactivating properties of these compounds. Together, these results support an inactivation mechanism involving Michael addition of the sulfur atom of active site cysteine 215 to the β-carbon of acrolein (Scheme 2).

In an effort to design highly selective activity based probes towards PTPs, Liu *et al.* screened aryl vinyl sulfonates and sulfones against several PTPs (Fig. 12).[123] Phenyl vinyl sulfone (PVS) and phenyl vinyl sulfonate (PVSN) inactivated the YopH PTP in a time- and concentration-dependent manner. These compounds showed comparable kinetics, modest affinity with K_I= 290 μM, k_{inact} = 0.061 min^{-1} (PVSN), and K_I = 350 μM, k_{inact} = 0.08 min^{-1} (PVS) against YopH. Similar kinetic results were observed with other PTPs such as PTP1B, PRL1, VHR, HePTP, DEP-1, and the low molecular weight PTP. Mass spectrometric and X-ray crystal structure analysis of PVS- and PVSN-modified YopH revealed covalent modification of active site Cys403 *via* a Michael addition of the thiolate of

Cys403 onto the β-carbon of the vinyl group (Fig. 13). The bond lengths for the C-S bonds (thioether) between Cys403 of YopH and PVS and PVSN are 1.82 and 1.73 Å, respectively, which are close to the typical C-S bond length of ~1.8 Å. X-ray crystal structures of PVS and PVSN modified YopH are very similar. Due to the presence of an extra oxygen atom, the SO_2 in PVSN moves 0.7 Å deeper into the active site; as a result, the H-bonding pattern varies between the two complexes. However, the total number of hydrogen bonds in both structures remains the same. PVSN showed no inactivation against acid or alkaline phosphatases. It was 240–700-fold less effective in inactivating the cysteine proteases, papain and cathepsin B compared to HePTP and VHR. The authors have developed activity-based probes using the PVSN moiety to profile PTP activity in complex proteome. They demonstrated the highly selective nature of these probes towards labeling PTPs from a complex *E. coli* proteome. These probes have several advantages over α-bromobenzylphosphonates, including higher affinity for the PTP active site, resistance to solvolysis, and increased cell-permeability.

A screen of the NIH molecular libraries small molecules repository (MLSMR) identified compounds 21 and 22 as covalent inhibitors of LYP (Fig. 14).[124] Kinetic analysis of these two compounds is consistent with covalent capture of the enzyme, with nanomolar K_I and reciprocal millisecond k_{inact} values, indicating the best k_{inact}/K_I ratios compared to other reported covalent LYP inhibitors. The anti-inflammatory compound BAY-11-7082 (Fig. 14) was recently reported as a potent, irreversible inhibitor of PTPs.[125] Molecular modeling studies and mass spectrometry analysis of BAY-11-7082 modified PTP1B, revealed covalent modification of the enzyme *via* Michael addition reaction between Cys215 thiolate and C2 carbon of the inhibitor (Fig. 15). Most importantly, this compound showed 100-fold selectivity toward classical PTPs over DUSPs. BAY-11-7082 caused an increase in tyrosine phosphorylation in RAW 264 macrophages, similar to the effects of vanadate, a general PTP inhibitor. These results indicate that many known reported biological effects of BAY-11-7082 in part may be due to its inhibition potential of PTP activity. The proposed mode of action for BAY-11-7082 is similar to that of PVSN and PVS.

Natural products serve as good starting points for the development of new drugs. Currently, several natural product derivatives have been reported as inhibitors of various disease relevant PTPs.[126–129] Ueda and co-workers identified 4-isoavenaciolide (4iA) (Fig. 14), a natural product isolated from fungal strain, as an irreversible inhibitor of a dual-specificity protein phosphatase, VHR (vaccinia H1 related) with an IC_{50} of 1.4 μM.[130] Mass spectrometry analyses of 4iA modified VHR fragments confirmed the covalent attachment of two molecules of 4-iA to one molecule of VHR at two different sites: Cys124 in the catalytic domain and Cys171 on the α6 helix positioned surface domain. Due to the presence of a reactive exo-methylene group in 4-iA, it was proposed that 4-iA inhibits VHR through a Michael addition of the catalytic cysteine (Cys124) thiol. Interestingly, 4-iA inhibited only dual-specific phosphatases and tyrosine phosphatases and showed no inhibition against serine/threonine phosphatases (PP1 and PP2A). These results indicate that 4-iA is a cysteine-targeting inhibitor of protein phosphatases with a common HCX_5RS/T motif in the catalytic site.

Menadione (Vitamin K_3) (Fig. 14), a naphthoquinone derivative, was shown to have antiproliferative activity towards a variety of human cells. This compound shows lower levels of toxicity compared to other quinone-based chemotherapeutic agents.[131] Menadione was found to inactivate CDC25A by covalent modification at the active site.[132] Therefore, Choi and co-workers screened a group of hydrophilic naphthoquinone derivatives against CDC25A. Analogue 2,3-dichloro-5,8-dihydroxynapthoquinone 23 (Fig. 14) was the most potent of the compounds tested. Flow cytometry revealed a cell-cycle delay at the G1/S phase transition, confirming a decrease in the CDC25A activity by 23. Later, Ham *et al.* synthesized a sulfone analogue of naphthoquinone 24 (Fig. 14) and tested it against a panel of PTPs.[133] Compound 24 inactivated PTP1B with a dissociation constant K_I of 3.5 μM and an inactivation rate constant k_{inact} of 2.2×10^{-2} sec^{-1}, while no inactivation was observed against CDC25A, CDC25B, CDC25C, YopH, and LAR (up to 40 μM). Dialysis and competitive experiments confirmed the active site directed irreversible inactivation of PTP1B by compound 24.

Trans-β-nitrostyrene (TBNS) and its derivatives were found to be slow-binding, reversible inhibitors of PTP1B, SHP1, and YopH.[134] In the absence of any added free thiols, compound 25 (TBNS) (Fig. 16) exhibited potent inhibition of PTP1B and YopH with an IC_{50} of 2.5 μM and 5 μM, respectively. It showed no inactivation against alkaline phosphatase. A structure-activity relationship of TBNS was established through the evaluation of a small collection of derivatives of TBNS. All the derivatives 26–32 (except 29) (Fig. 16) inactivated PTP1B with IC_{50} values ranging from 2.7 μM to 28 μM in the absence of added thiols. Interestingly, compound 29 showed no significant inhibition of PTP1B and SHP1, even at higher concentrations up to 600 μM. This suggests the necessity of C=C bond in TBNS, C=C maintains a rigid planar geometry that is critical for binding and/or positioning the NO_2 group for nucleophilic attack by the active site thiolate. Analogue 33, which has a tripeptide (Gly-Glu-Glu) at the para position of TBNS, showed slightly improved potency with as IC_{50} of 1.4 μM against PTP1B. UV-vis spectroscopy, site-directed mutagenesis, and heteronuclear single-quantum correlation (HSQC) NMR spectroscopy studies suggested a mechanism in which TBNS fits into the active site, in a manner similar to that of pTyr, followed by nucleophilic attack of Cys-215 thiolate on to the nitro group in TBNS to form a covalent thiol-reversible adduct (Fig. 17).

5. Carbonyl compounds

Peptidyl aldehydes are known to inhibit serine and cysteine proteases *via* a covalent, reversible mechanism.[135] These aldehydes react with active site nuclephiles in proteases to form a covalent hemiacetal or hemithioacetal linkage that mimics the transition state of a normal enzyme reaction. This tetrahedral hemiactal or hemithioacetal intermediate can collapse to regenerate the free enzyme. As a result, the enzyme-inhibitor complex is in equilibrium with free inhibitor and free enzyme. By taking advantage of this reversible covalent nature of the reaction between aldehydes and protein nucleophiles, medicinal chemists have developed a number of highly specific aldehyde-based inhibitors for proteases. Interestingly, calpeptin, a potent dipeptide aldehyde inhibitor of calpain, showed modest reactivity over several PTPs.[136,137] This observation led Pei *et al.* to screeen a set of aldehydes against PTPs such as PTP1B, SHP1, and VHR.[138] Among the aldehydes tested

(34–36) (Fig. 18), 4-carboxycinnamaldehyde 36 showed the highest activity against PTPs, with IC_{50} values of 230 and 970 μM against SHP1 and PTP1B, respectively. Interestingly, it showed no inhibition against (up to 2 mM) VHR, which may be due to a wider, shallower active-site of VHR compared to other two PTPs.[139] Early studies by Moran *et al.* demonstrated that a weakly active cinnamic acid can be converted into a highly potent inhibitor 37 (Fig. 18) for PTP1B ($K_I = 79$ nM) by introducing the tripeptide (Gly-Glu-Glu) at the para position of cinnamic acid.[140] In an attempt to improve potency and selectivity of 36, Pei *et al.* used Moran's strategy and attached the tripeptide GEE at the para position of cinnamaldehyde to synthesize 38 (Cinn-GEE). As expected, aldehyde 38 showed improved potency against all three PTPs (e.g., $K_I = 5.4$ μM against PTP1B). The mechanism of inhibition was investigated using ^{1}H-^{13}C HSQC NMR spectroscopy of ^{13}C labelled Cinn-GEE and site-directed mutagenesis. HSQC of ^{13}C labeled aldehyde carbon containing Cinn-GEE showed a single cross-peak at δ 9.64 (^{1}H) and δ 201 (^{13}C), whereas the PTP1B/Cinn-GEE complex showed three distinct cross-peaks at δ 7.6–7.8 (^{1}H) and 130–137 (^{13}C). Mutation of the catalytic cysteine to alanine (C215A) showed no effect on the crosspeaks, but mutation of active-site arginine to alanine (R221A) abolished all three cross-peaks. Similar experiments with ^{13}C labeled benzylic carbon containing a Cinn-GEE/PTP1B complex revealed a change in the hybridization from sp^2 to sp^3 for the benzylic carbon. This is consistent with a mechanism involving the formation of an initial noncovalent (E•I) complex, followed by a covalent imine adduct (E**-**I) formation between the guanidine group of the active-site Arg221 and aldehyde of the inhibitor (Fig. 19). Conjugate addition at the β-carbon by an unidentified nucleophile (could be β-mercaptoethanol or a water molecule in buffer or protein side chain) produced the enamine (E**-**I*).

In an another instance, Shim and co-workers screened a set of over thirty formylchromone derivatives against PTP1B.[141] Their search revealed TP_{33} and TP_{34} (Fig. 18) as the most potent and selective inhibitors for PTP1B. Both TP_{33} and TP_{34} showed 500, 30, and >10-fold selectivety over LAR, TC-PTP, and SHP1 respectively. Molecular modeling studies of TP_{34} on PTP1B placed the aldehyde carbon atom 3.4 Å away from the sulfur atom of Cys215 and the nitrogen atom of Arg221, suggesting the posibility of forming S–C bond (thioether) or N=C bond (imine). The S atom was oriented in the right position to the carbonyl of aldehyde allowing a nucleophilic attack, whereas Arg221 was not in a proper orientation for imine formation. These observations favor the formation of thioether linkage over imine, suggesting a mechanism different from the one reported by Pei and co-workers.

6. Isothiocyanates

Dietary isothiocyanates (ITCs), such as allyl isothiocyanate (AITC) and phenethyl isothiocyanate (PEITC) (Fig. 20A), have anti-carcinogenic activities and are of potential use in the chemoprevention of cancer.[142–144] More recently, Lewis *et al.* reported thiol reversible inactivation of PTP1B and SHP2 with various isothiocyanates.[145] Results in the case of PTP1B confirmed a mechanism involving covalent, thiol reversible, modification of the active site cysteine residue. Second-order inactivation rate constants for inactivation of SHP2 by AITC were reported as 16 and 9 M^{-1} s^{-1} in the Tris and glutarate buffer systems, respectively. The presence of active site directed inhibitors, such as phosphate and arsenate, decreased the inactivation rates at which SHP2 was inactivated by AITC or sulforapane,

indicating covalent modification of the enzyme's active site (Fig. 20B). PTP1B dephosphorylates Tyr-490 of TrkA in PC12 cells, suggesting its key regulatory role in NGF signaling. Over-expression of PTP1B in PC12 cells resulted in inhibition of neuritogenesis due to the dephosphorylation of TrkA. 6-methylsulfinylhexyl isothiocyanate (6-HITC), the major ITC compound in wasabi, improved neuritogenesis in PC12 cells by inhibiting PTP1B.[146] Unfortunately, a detailed mechanism of regulation of PTP1B by 6-HITC was not reported. These results suggest that 6-HITC may serve as a potential lead compound in the development of drugs to treat neurodegenerative diseases.

7. Epoxides

Due to their strained three-membered ring structures, epoxides (oxiranes) offer reactive electrophilic carbon atoms that can be attacked by a variety of nucleophiles. In order to identify the active site residues of low molecular weight PTP, Zhang and co-workers conducted inactivation experiments using epoxides such as phosphomycin, 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), and (R)-(−) and (S)-(+)-2-(benzyloxymethyl)-oxiranes (Fig. 21).[147] EPNP and (R)-(−) and (S)-(+)-2-(benzyloxymethyl)-oxiranes acted as active-site directed irreversible covalent inactivators, whereas phosphomycin was a simple competitive inhibitor of the enzyme. These observations were consistent with the importance of a hydrophobic moiety on the substrate for efficient binding. An investigation into the mechanism of inactivation by EPNP revealed covalent binding of Cys62 and Cys145 residues on the low molecular weight PTP. Since these two Cys residues are away from the active site, the mode of inhibition by EPNP may be allosteric.

In an attempt to further develop epoxides as PTP inactivators, Park *et al.* tested styrene oxide against a panel of five PTPs: the catalytic domain of human SHP1 (SHP1-cat), a yeast PTP1 (YPTP1), a *Yersinia* PTP (YopH), the cytosolic PTP domain of CD45 (CD45-cyto), and human PTP1B.[148] In the case of PTP1B and SHP1-cat, over 80% of inhibition was observed when the enzyme was preincubated with 20 mM styrene oxide for 10 min in a *p*-nitrophenyl phosphate (pNPP) based assay. Further experiments with PTP1B at various preincubation times and inhibitor concentrations confirmed the time and concentration-dependent inhibition. Preincubation of 2 mM of compounds 39, 40, 41, and 42 (Fig. 21) for 10 min with PTP1B showed 85%, 81%, 68%, and 29% of remaining activity, respectively. Compound 42 with a naphthalene ring showed improved inhibition, but poor solubility prevented reliable measurement of kinetic data at higher concentrations. Later, Dana and coworkers synthesized a small set of sulfonyloxiranyl derivatives based on molecular modeling studies.[149] Reaction of compounds 43–47 (Fig. 21) with PTP1B under pseudo first order kinetic conditions ([43] ≫ [PTP1B]) resulted in a time dependent loss of phosphatase activity. The covalent nature of inactivation was further confirmed by dilution, competition, and MALDI-TOF mass spectrometry experiments.[150] Epoxides 43, 44, and 45 showed very similar reactivity; epoxide 46 showed slightly more reactivity, whereas 47 showed no reactivity against PTP1B. The lack of reactivity of aliphatic analog 47 with PTP1B indicated the importance an aryl moiety for effective PTP1B binding. In human A549 lung cancer cell lines, compound 39 caused increase in intracellular tyrosine phosphorylation levels in a dose-dependent manner, suggesting a cell-permeable nature of this class of compounds.

8. Other PTP inactivation mechanisms

In addition to the above discussed covalent inhibitors, several other endogenous and exogenous chemicals have also been reported as inactivators of PTPs. Recently, new covalent allosteric inhibition of PTPs was also reported where covalent modification of cysteines present in pockets close to the active site led to loss of phosphatase activity.[151] Fluorogenic reagent 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABDF, compound 48) (Fig. 22) was reported as an allosteric inhibitor of PTP1B.[152] Tryptic digestion followed by mass spectrometry analysis confirmed covalent modification of Cys121 in PTP1B. The authors reasoned that disruption of the active-site, due to the covalent modification of Cys121, was responsible for the loss of phosphatase activity. Cys 121 is a conserved residue among various PTPs, and ABDF inactivates closely related TC-PTP and LAR, but not CD45, showing some degree of sequence specificity toward inactivation. Surprisingly, ABDF reacts selectively with only cysteine 121, leaving unmodified five other cysteines, including the highly reactive active site Cys215 in PTP1B. In addition, ABDF caused a dose-dependent increase in the level of insulin receptor phosphorylation in CHO-hIR cells through PTP1B inhibition. Similar allosteric inhibition of PTP1B was observed with 1,2-naphthoquinone 49 (Fig. 22).[153] MALDI-TOF mass spectrometry analysis and mutation experiments revealed that compound 49 mediated PTP1B inactivation was mainly due to covalent modification of Cys-121 in the enzyme and some binding interactions to His-25 and Cys-215. These two examples, together with that of EPNP and LWM-PTP,[147] illustrate that targeting cysteine residues present in pockets outside an active site may also be exploited in designing inhibitors for PTPs.

The benzopentatiepin analogue 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride (TC-2153) was identified as a reversible covalent inhibitor of STriatal-Enriched protein tyrosine Phosphatase (STEP).[154] *In vitro* selectivity experiments on full length PTPs revealed that TC-2153 is more potent on isoforms of STEP, $STEP_{46}$ ($IC_{50} = 57$ nM) and $STEP_{61}$ ($IC_{50} = 93$ nM), compared to highly related PTPs, PTP-SL ($IC_{50} = 220$ nM), and HePTP ($IC_{50} = 363$ nM). It showed even greater selectivity over SHP2 ($IC_{50} = 6,896$ nM) and PTP1B ($IC_{50} = 723$ nM). In cell-based assays, TC-2153 increased tyrosine phosphorylation of STEP substrates ERK1/2, Pyk2, and GluN2B. Kinetic constants k_{inact} (0.0176 s^{-1}), K_I (115 nM), and k_{inact}/K_I (153,000 $M^{-1}s^{-1}$) for TC-2153 against STEP were reported.[68] TC-2153 treatment restored BDNF expression and improved cognitive and motor functions in phencyclidine-treated mice through inhibition of $STEP_{61}$.

The seleninic acid group ($RSeO_2H$) is a bioisostere for carboxylic acid (RCO_2H), due to its similar structural and protolytic properties.[155] Monoanionic seleninate is also considered as a potential bioisosteric match for the phosphate group.[156,157] In 2008, Abdo *et al.* synthesized compound 50 (Fig. 22) with the seleninate acting as a phosphate mimic in pTyr.[158] Incubation of 50 with YopH, PTP1B, VHR, and VHX resulted in a time and concentration dependent loss of enzyme activity. Kinetic, mutational, and mass spectrometric analytical data together revealed 50 as an irreversible inhibitor of the PTPs tested. YopH inactivation by 50 showed saturation kinetics and exibited values for the binding constant K_I and the inactivation rate constant k_i of 44.5 μM and 0.243 min^{-1}, respectively. Further analysis with X-ray crystalographic structure data of PTP1B complexed

with 50, confirmed the covalent modification of PTP1B *via* a selenosulfide bond between the sulfur atom of Cys215 and the selenium in 50 (Fig. 23). Selenosulfide bonds can be readily cleaved by the action of thiols (DTT) in aqueous solution.[159] Interestingly, the selenosulfide linkage present in modified YopH is stable even at millimolar concentrations of DTT, suggesting the inaccesible nature of this bond in the deep active-site of YopH.

In 2010, Piovan *et al.* screened a series of twelve hypervalent selenium (IV) and tellurium (IV) compounds (organoselenanes and organotelluranes) as potential inactivators of PTPs.[160] All these compounds (Fig. 24) inactivated PTP1B and YopH in a time and concentration dependent manner. The inactivation is most likely due to the covalent modification of the active site cysteine thiol by the chalcogen atom. Kinetic analysis of these compounds (51–62) revealed organotelluranes as being more potent than organoselenanes for inactivating PTP1B and YopH. SAR studies showed no obvious contributions from halogens or stereochemistry at the benzylic position on inactivation power of these organochalcogenanes. Organotellurane 57 showed an equilibrium binding constant K_I of 1.9 $\pm$ 0.17 mM and an inactivation rate constant k_{inact} of 17.2 $\pm$ 0.9 min^{-1}. Though these compounds are weak inactivators of the PTPs tested, potent and selective inhibitors could be developed by introducing specificity determinants into the aryl ring.

Heavy metals have been noted as covalent inhibitors of various PTPs. Kim and co-workers examined the regulatory roles of heavy metals ions such as Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} on human dual specificity protein phosphatase VHR *in vitro*.[161] Among the metal ions examined, only Fe^{3+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} inactivated VHR, and Cu^{2+} ion demonstrated the most potent inactivation. The addition of excess EDTA diminished Fe^{3+}, Zn^{2+}, and Cd^{2+} mediated inactivation of VHR, whereas additional DTT was required to restore the activity of Cu^{2+}-inactivated VHR. These two observations suggest that inactivation by Fe^{3+}, Zn^{2+}, and Cd^{2+} occurred largely from simple binding of these metal ions to certain residues of VHR; however, the Cu^{2+} mediated inactivation was largely due to the oxidative property of Cu^{2+}. Additionally, Cu^{2+} inactivated other PTPs such as SHP1 and PTP1B. In a recent study, Karvar *et al.* demonstrated Au(I)-phosphine complex, [Au((CH$_2$CH$_2$CN)$_2$PPh)Cl], as a selective LYP inhibitor both *in vitro* and in cells.[162] The complex 63 showed an IC$_{50}$ of 3.5 μM against LYP, and over 14 fold selectivity against PTP-PEST, HePTP, and CD45. Mode of inhibition studies on LYP, the LYP mutant C129/131S, and PTP-PEST suggested the coordination of Au(I) by both the active site cysteine (Cys227) as well as either Cys129 or Cys231 as a potential mechanism for selective LYP inhibition. This is consistent with previous reports on competitive and thiol reversible inhibition of PTPs by Au(I) complexes.[163,164] In an interesting approach, Bishop and co-workers introduced non-natural allosteric-inhibition sites in PTPs through protein engineering; these sites assist to sensitize target PTPs to selective inhibition by FlAsH (fluorescein arsenical hairpin binder) and other biarsenical compounds (Fig. 25).[165] They designed these sites based on naturally occurring cryptic allosteric site present in a wild-type SHP2 catalytic domain that is sensitive to inhibition by biarsenicals even in the absence of engineering.[166,167] To improve interactions between SHP2's allosteric site and FlAsH, they introduced a cysteine residue at position 368 (V368C mutant), because valine 368 is not conserved among classical PTPs, and it also contains a solvent-accessible side chain. As expected, V368C SHP2's demonstrated increased affinity of the protein/FlAsH interaction

through an additional covalent interaction between the thiol of cysteine 368 and arsenic. Moreover, the authors successfully introduced these allosteric-inhibition sites into other PTPs including PTP1B, HePTP, PTPH1, and FAP1 from different PTP subfamilies, to generate biarsenical responsive PTPs.

Dithiolethiones are known for their chemopreventive activity.[168,169] Bhattacharyya *et al.* demonstrated that the dithiolethione analogue oltipraz inactivated PTPs *via* covalent, but thiol-reversible, modification of catalytic cysteine residues.[170] The proposed mechanism involves the nucleophilic attack of thiolate of Cys215 followed by a series of thiol disulfide exchange reactions which then leads to the covalent modification of Cys215 of the PTP1B (Fig. 26). It was shown that the UV irradiation of PTP-overexpressing cells leads to partial inactivation of several PTPs. Four PTPs (SHP1, RPTPα, RPTPσ, and DEP-1), were partially inactivated by UV irradiation at 355 nm. In the case of RPTPα, UV irradiation leads to the generation of a substrate-trapping configuration that is similar to the C433S mutant.[171]

As mentioned in the previous section, oxidation of PTPs by various endogenous chemicals produce unique electrophilic groups such as sulfenic acid and sulfenyl amide, which could be captured by nucleophilic reagents to completely knock down the phosphatase activity of PTPs. For instance, Kate Carroll's group recently reported dimedone derivatives as covalent inhibitors of the sulfenic acid forms of YopH and PTP1B (Fig. 27A).[172–174] Classical tyrosine phosphatases, PTP1B and PTPα when exposed to biological oxidants, form an electrophilic sulfenyl amide *via a* sulfenic acid intermediate. Chemical model studies conducted by Shieu *et al.* and Ruddraraju *et al.* showed that the sulfenyl amide moiety in PTPs could be covalently modified by 1,3-dicarbonyl compounds to generate stable adducts (Fig. 27B).[175–177] These adducts were shown to be stable under high concentrations of thiols (e.g., 50 mM DTT at 45 °C for 6 h). In an another study, Parson *et al.* reported thiol reversible covalent capture of sulfenyl amide by sulfone stabilized carbon nucleophiles such as β-ketosulfones, β-disulfones, and β-cyano sulfones.[178] Both sulfenic acid and sulfenyl amide present a unique electrophilic sulfur atom for selective covalent modification of PTPs by a suitable nucleophile. Taken together, these results suggest that the above mentioned nucleophilic warhead groups, when incorporated into a high affinity binding module, may generate compounds that exploit non-covalent interactions to facilitate selective irreversible capture of oxidized PTPs. The ability to target differentially oxidized cysteine residues on PTPs may afford further means of achieving selectivity and offer an alternative strategy to modulate PTP activity in cells.

Conclusion

Given the diverse regulatory roles played by PTPs and their implications in human diseases, such as cancer, diabetes, and neurological disorders, PTPs are highly important drug targets. But, developing potent, selective, and active site-directed small molecule inhibitors (with drug-like properties) to target PTPs is challenging, mainly due to the highly conserved and cationic nature of the active sites of various PTPs. However, several research groups have successfully generated active site-directed small molecule inhibitors with high potency and selectivity as well as robust *in vivo* activity to target disease-relevant PTPs.[10, 11] Currently, there is also renewed interest among investigators to develop allosteric and covalent

inhibitors to modulate PTP activity. Allosteric inhibition strategy has generated a few successful small molecule inhibitors in the case of PTP1B and SHP2. The covalent inhibition strategy was successful in the case of PTKs drug discovery and has generated some promising small molecules for selective inactivation of PTKs.[34] But, no selective inactivators have been reported for an individual PTP. Covalent inhibitors have several advantages over traditional non-covalent inhibitors; low doses are needed to show the desired pharmaceutical effect, generate stable and long-lived ties, and are resistant to mutations.[179] Although off-target interactions are a major concern, a careful and systematic approach could lead to a drug candidate that addresses the above-mentioned challenges in PTP drug discovery. We have summarized the efforts undertaken by researchers to design covalent inhibitors for selective inactivation of PTPs. Some of these inhibitors demonstrated class specific inhibition; however, none of them showed selectivity toward an individual PTP. We believe that an important future direction for the field is to develop selective covalent PTP inhibitors for functional interrogation, target validation, and therapeutic development.

One approach to install specificity for covalent inhibition of PTPs is to design bifunctional molecules that consist of a specificity determinant and a functional warhead capable of generating a covalent linkage with the PTP of choice. The former serves as a template by binding to the target PTP, thereby allowing the latter to undergo covalent modification of an active site residue. Although the active site structures are highly conserved among various PTPs, the surfaces of these proteins are remarkably diverse, a property that may be responsible for substrate recognition, regulatory mechanisms such as oligomerization, and association with other regulatory proteins.[180] Therefore, targeting non-conserved surface residues for achieving selectivity using the exo-affinity labelling technique is also a viable strategy for the inactivation of individual PTPs.[181] This strategy involves a combination of an inhibitor and an electrophile, where an inhibitor binds to the active site and the electrophile extends out of the active-site and covalently modifies a non-conserved surface residue on the target PTP. Covalent modification of post-translationally modified PTPs, such as oxidized or nitrosylated forms, also has great potential for future drug discovery research. The success of covalent inactivation of oxidized forms of PTPs depends on a better understanding of mechanisms leading to selective PTP oxidation in a cellular environment. The strategy of introducing an electrophile onto a highly potent and selective inhibitor of a particular target PTP, based on X-ray crystal structure studies or docking studies, could provide selective and potent inactivators of PTPs. Computational tools such as CovalentDock,[182] GOLD,[183] CovDock-VS,[184] Autodock,[185] Glide,[186] CovDock,[187] etc. are available to aid researchers to design covalent inhibitors with drug-like properties.[188] Covalent inhibitors can also be used to develop chemical and biological tools, such as activity-based probes (ABPs), for functional assignment of PTPs at the proteome level in both normal and diseased states.

Acknowledgments

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Biographies

Kasi V. Ruddraraju was born in Andhra Pradesh, India. He received his Master's degree in organic chemistry from Osmania University in 2007. Then he joined Albany molecular research Inc. (AMRI) as a research scientist. He moved to the United States in 2011 to pursue his PhD degree in organic chemistry under Dr. Kent Gates at the University of Missouri-Columbia. His PhD thesis research involved design, synthesis, and structure activity relationship (SAR) studies of covalent inhibitors for protein tyrosine phosphatase 1B (PTP1B). Currently, he is working as a post-doctoral research associate for Dr. Zhong-Yin Zhang in the department of medicinal chemistry and molecular pharmacology at Purdue

University. His current research involves design and synthesis of therapeutic agents that target protein tyrosine phosphatases (PTPs) to treat diabetes and various cancers.

Dr. Zhong-Yin Zhang is Distinguished Profesor of Medicinal Chemistry, Robert C. and Charlotte P. Anderson Chair in Pharmacology, and Head of the Department of Medicinal Chemistry and Molecular Pharmacology at Purdue University. Research in his laboratory spans the disciplines of chemistry and biology with an emphasis on the structure and function of protein tyrosine phosphatases (PTPs), roles of PTP in normal physiology and pathological conditions, and the design and synthesis of PTP inhibitors as chemical probes to interrogate PTP function and as novel therapeutics for the treatment of cancer, diabetes and obesity, autoimmune disorders, neurodegenerative and infectious diseases. He obtained his Ph.D. in Chemistry from Purdue University and completed his postdoctoral training at the University of Michigan. Before returning to Purdue, he was Robert A. Harris Professor and Chairman of the Department of Biochemistry and Molecular Biology at Indiana University School of Medicine.
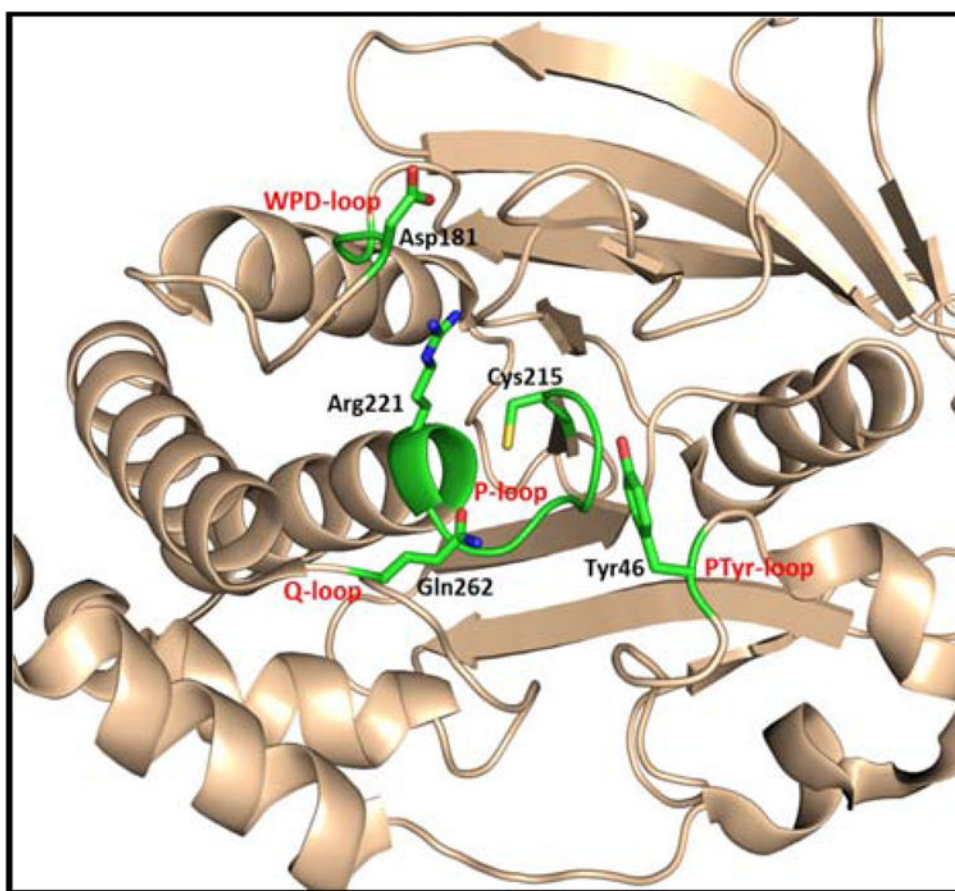
Fig. 1.
X-ray crystal structure of PTP1B showing the key residues for catalytic activity.
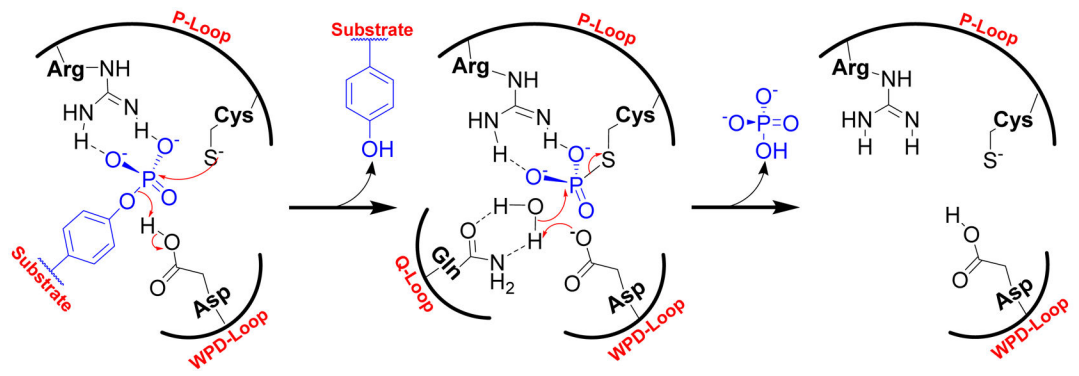
Fig. 2.
The general mechanism of the PTP catalyzed dephosphorylation reaction.
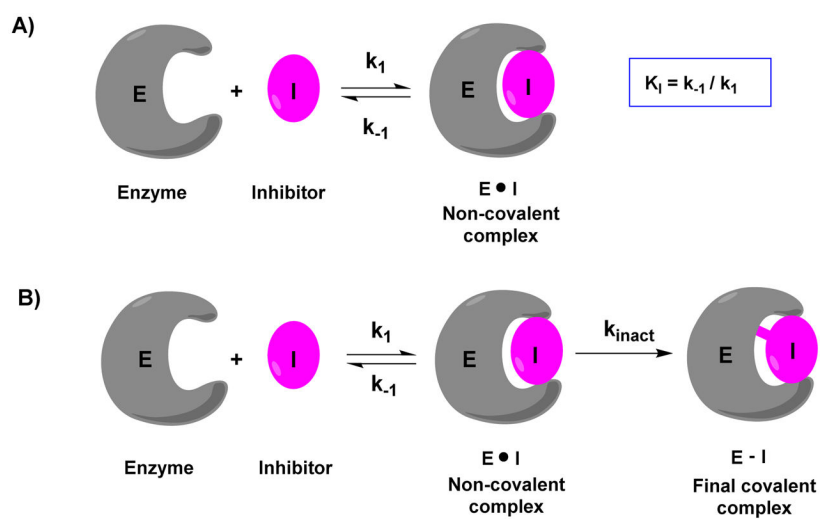
Fig. 3.
Comparison of traditional non-covalent and covalent interactions between an enzyme and an inhibitor. A) Interaction of traditional non-covalent reversible inhibitor with an enzyme. B) Interaction of covalent inhibitor with an enzyme.
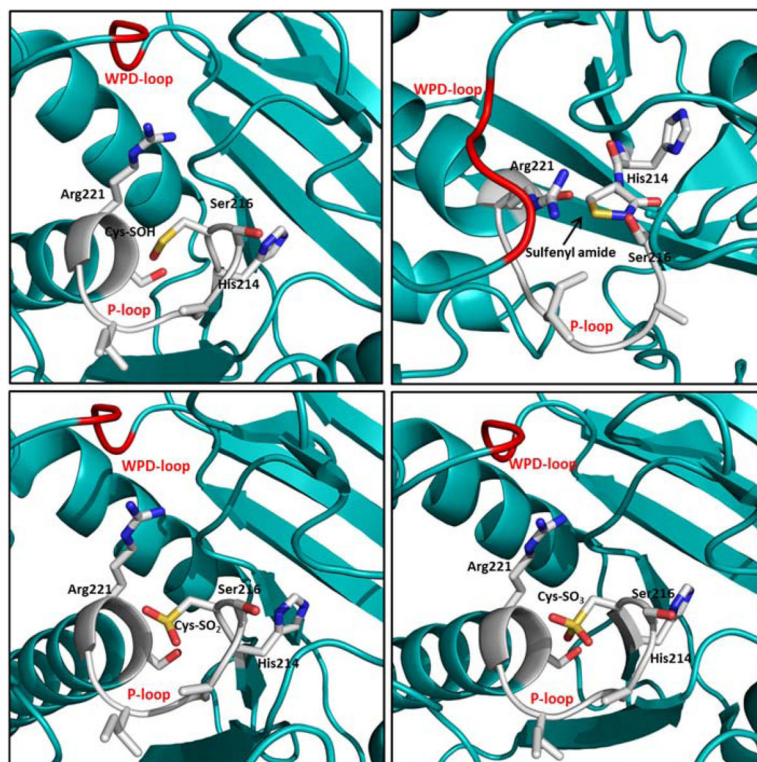
Fig. 4.

X-ray crystal structures of oxidized forms of PTP1B, P-loop residues shown in ball and stick and WPD loop in red. A) Sulfenic acid form (pdb code: 1oet).[83] B) Sulfenyl amide form (pdb code: 1oes).[83] C) Sulfinic acid form (pdb code: 1oeu).[83] D) Sulfonic acid form (pdb code: 1oeo).[82]
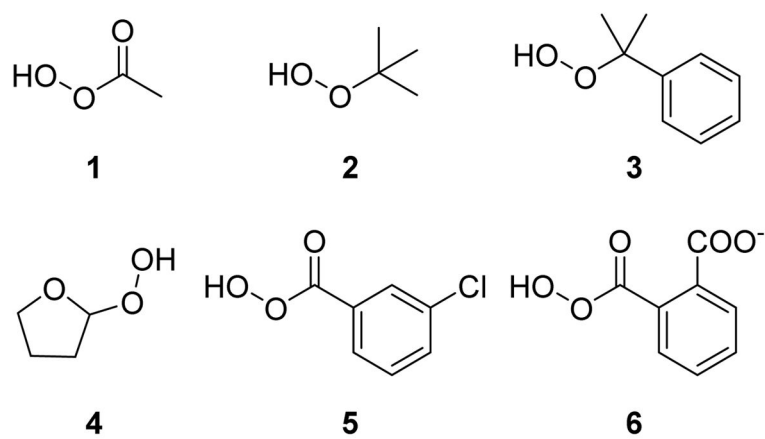
Fig. 5.
Chemical structures of organic hydroperoxides used for PTP1B inactivation. [93]
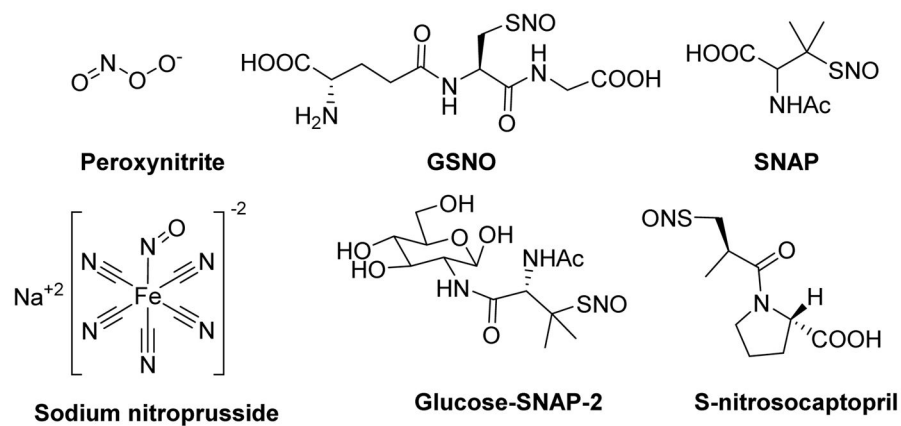
Fig. 6.
Structures of various nitroso compounds used for inactivation of PTPs.[100]
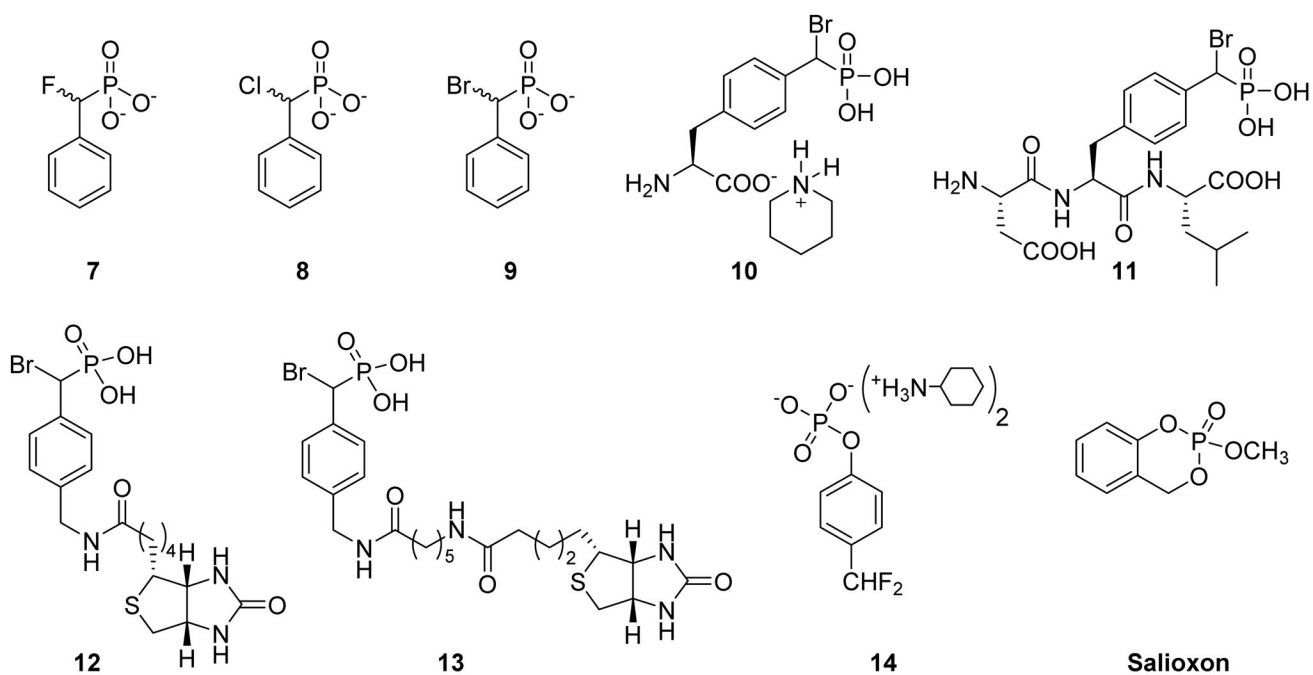
Fig. 7.
Structures of phosphate and phosphonate based covalent inhibitors.

A)

B)
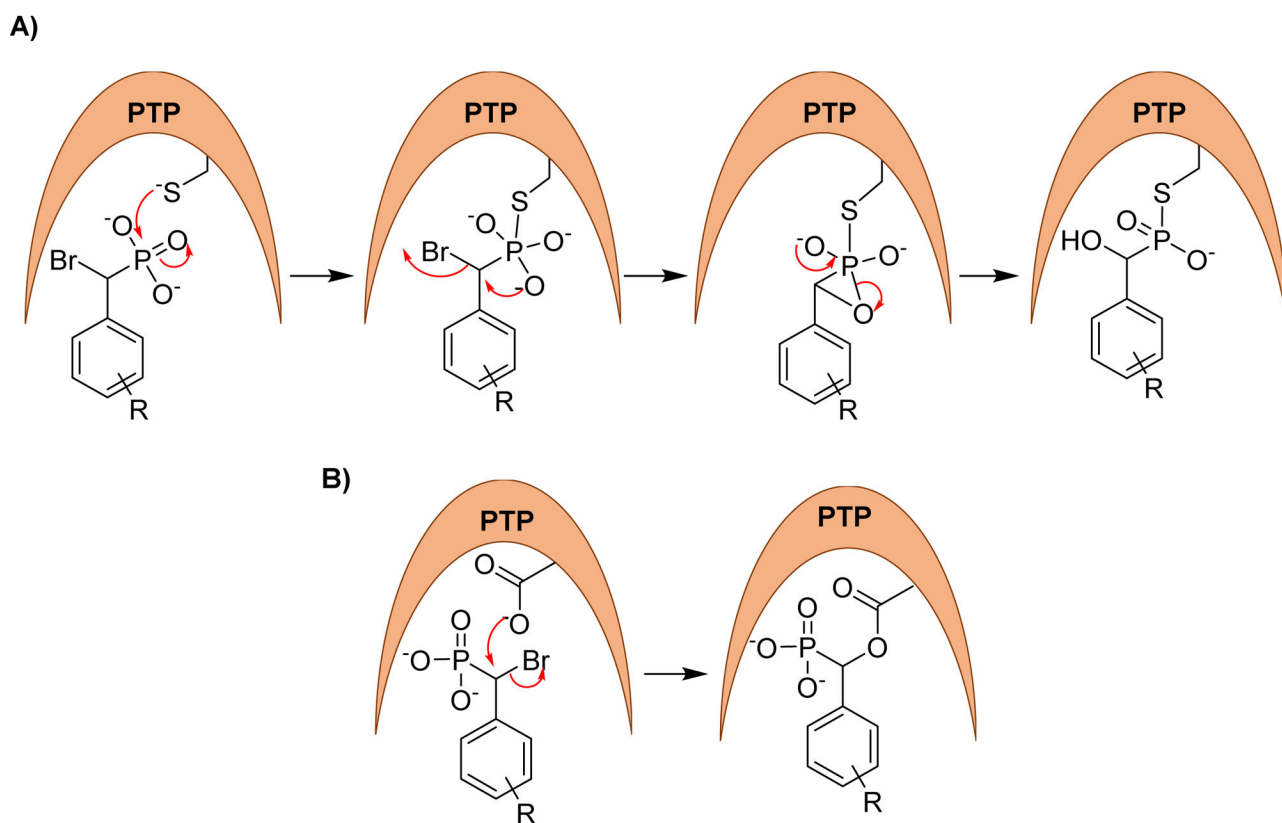
Fig. 8.
Proposed mechanism for covalent modification of PTPs by α-bromophosphonates.
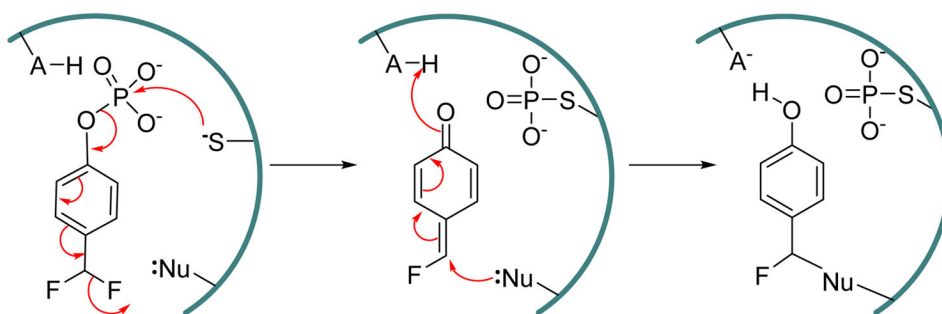
Fig. 9.
Proposed mechanism of inactivation of PTPs by 4-difluoromethylphenyl phosphate (DFPP).
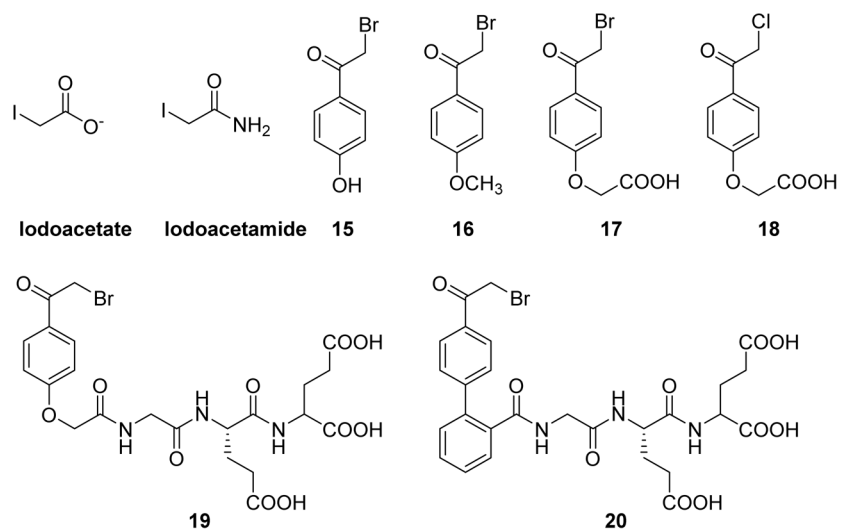
Fig. 10.
Chemical structures of α-halo ketones used as inactivators of PTPs.
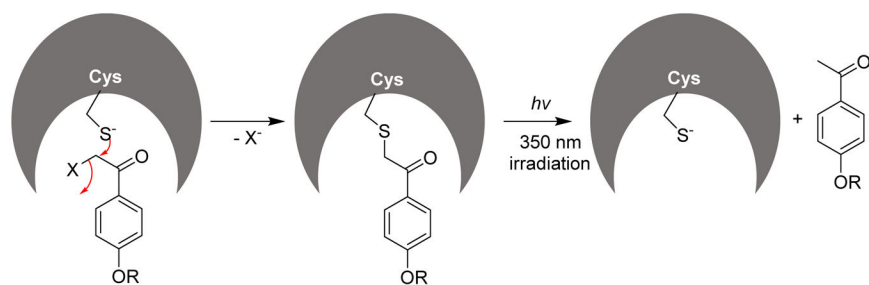
Fig. 11.
Inactivation of PTPs by α-haloacetophenones.
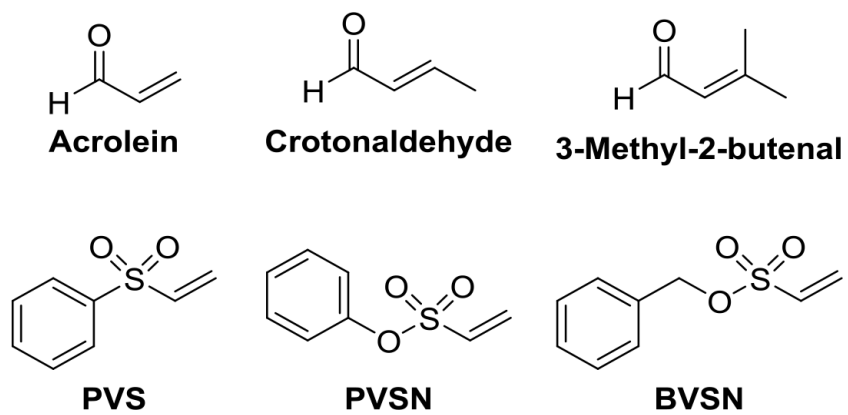
Fig. 12.
Structures of α,β-unsaturated compounds used for covalent modification of PTPs. [122, 123]
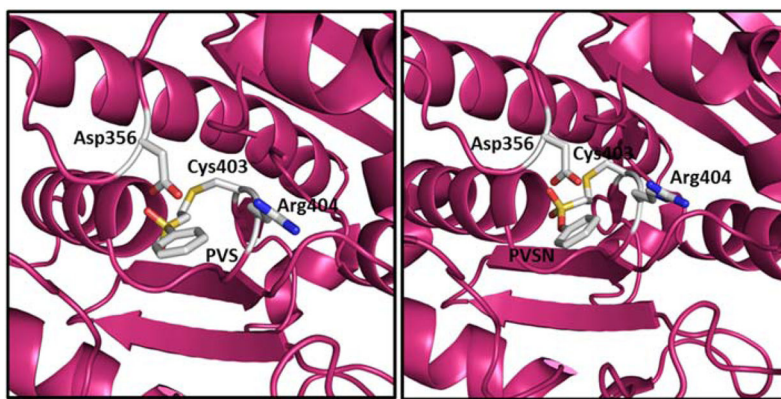
Fig. 13.
X-ray crystal structures of PVS (left) and PVSN (right) complexes with YopH. [123]
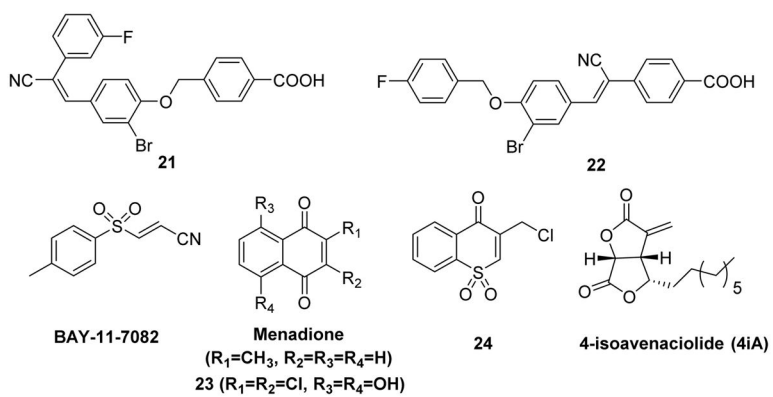
Fig. 14.
Structures of electrophilic compounds with activity as covalent PTP inhibitors.
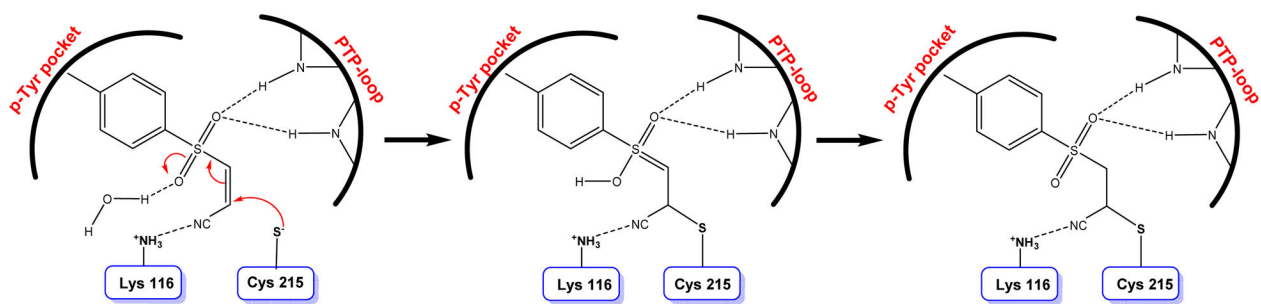
Fig. 15.
Proposed mechanism for the inactivation of PTP1B by BAY-11-7082.

25 (R = H)
26 (R = CH$_3$)
27 (R = OCH$_3$)
28 (R = CO$_2$H)

29

30

31

32

33
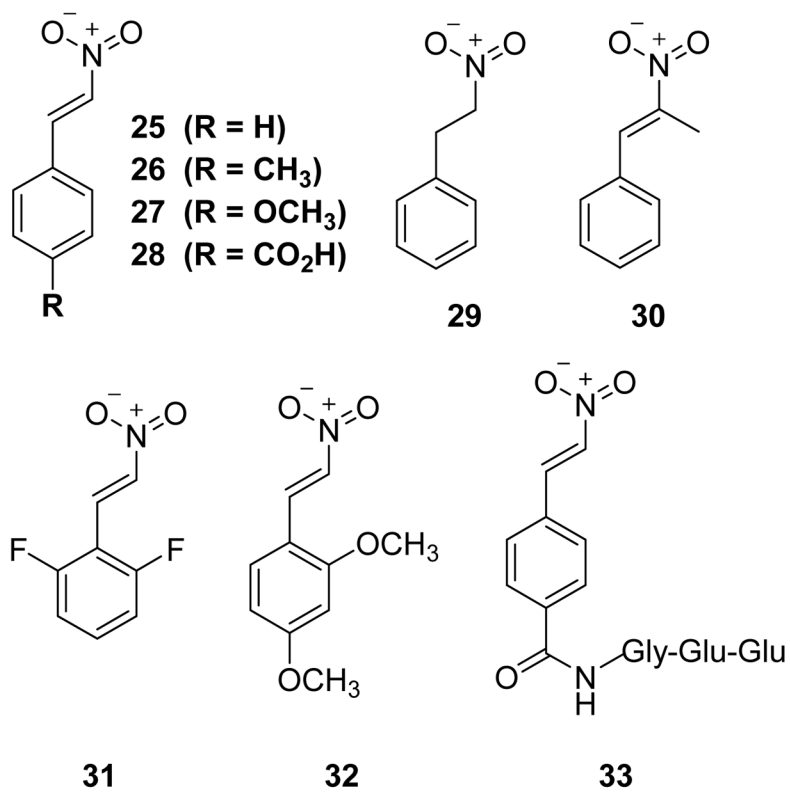
Fig. 16.
Chemical structures of PTP inactivators with nitro group.
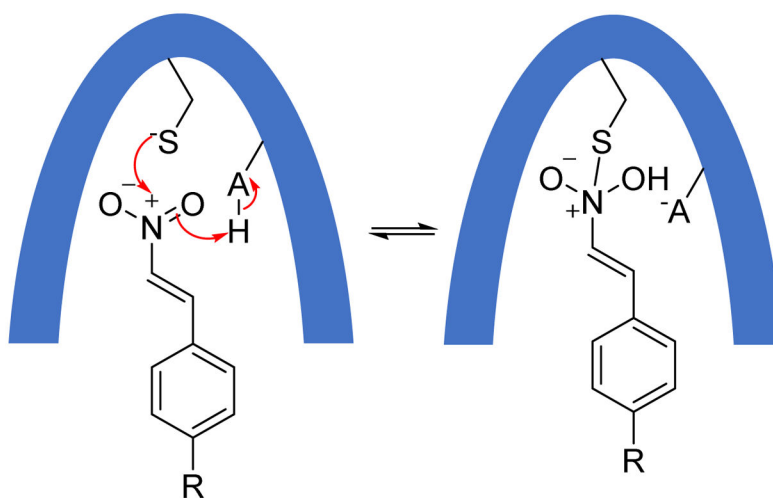
Fig. 17.
Reversible covalent modification of PTPs by TBNS derivatives.
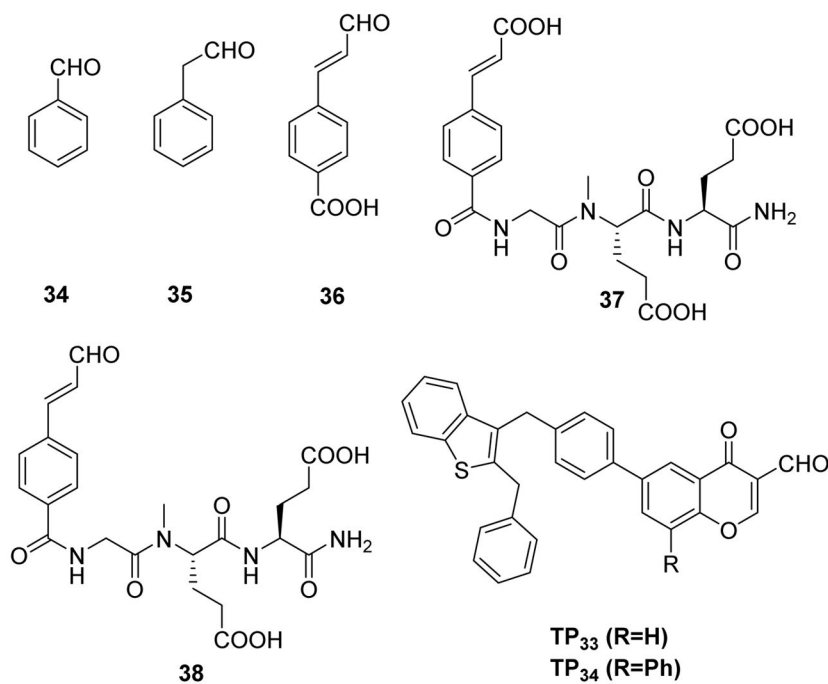
Fig. 18.
Structures of aldehyde based inhibitors used for inactivation of PTPs. [138, 140, 141]
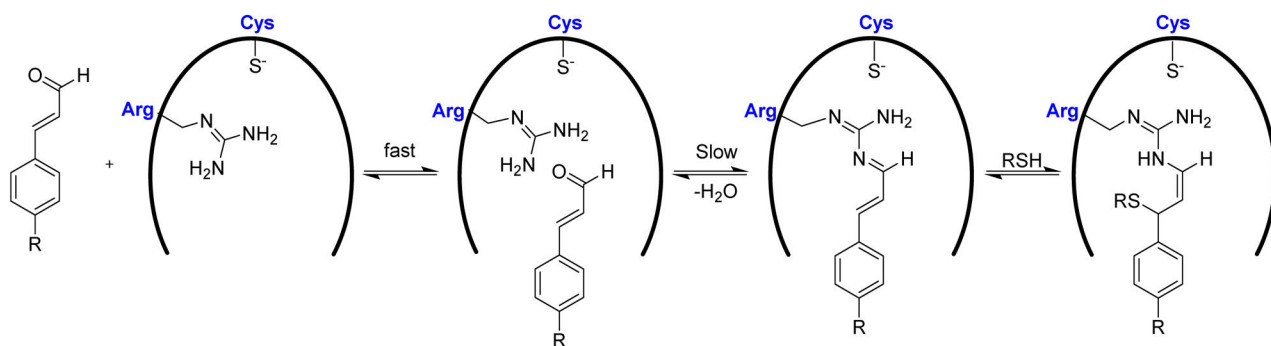
Fig. 19.
Proposed mechanism for the reaction of PTP with cinnamaldehyde derivatives (RSH = mercaptoethanol).
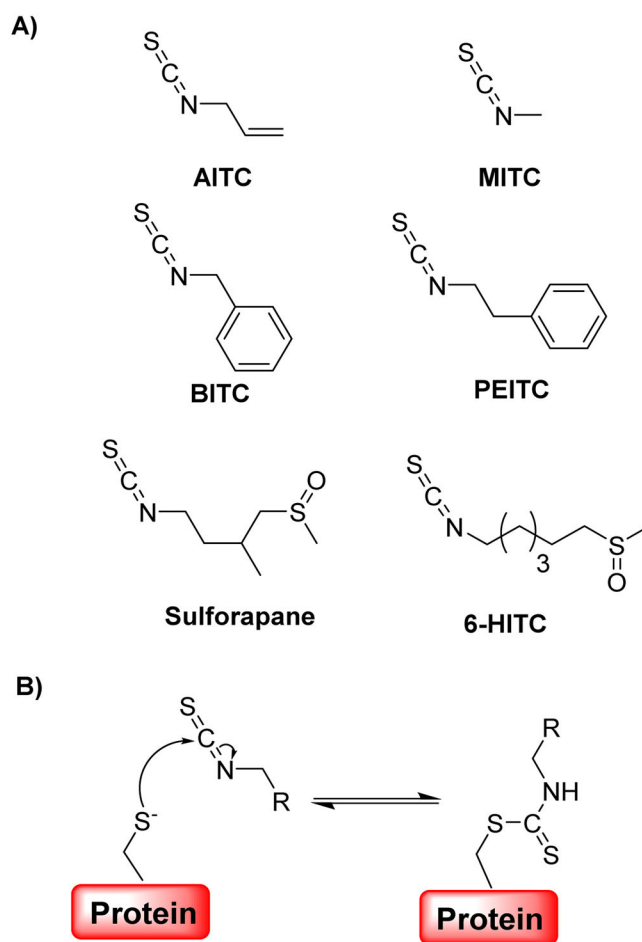
Fig. 20.
A) Structures of various isothiocyanates used by Lewis *et al.* and Shibata *et al.* as
inactivators of PTPs. B) Proposed mechanism for the reaction between isothiocyanates and
cysteine residues in proteins.
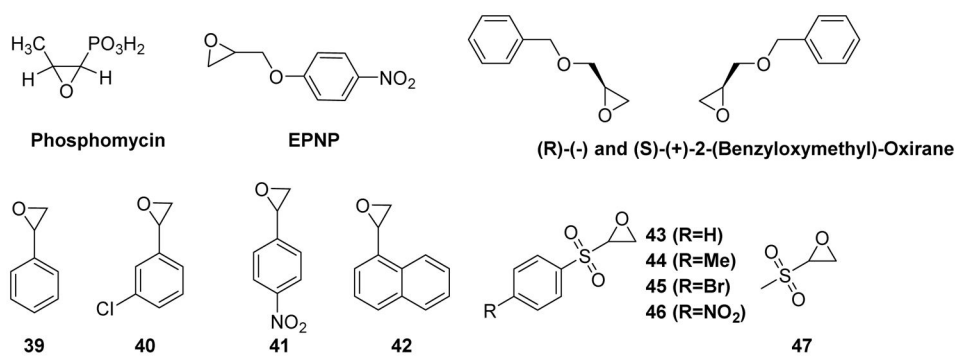
Fig. 21.
Chemical structures of epoxides used to inactivate PTPs.
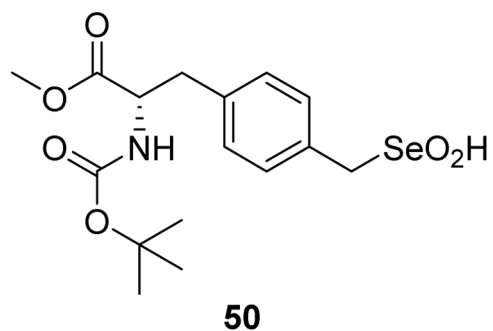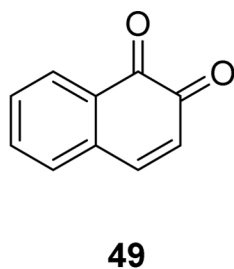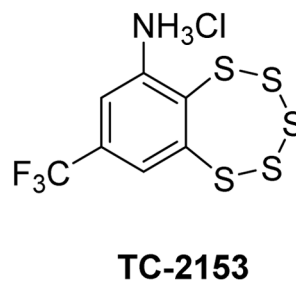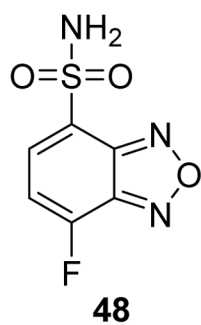
Fig. 22.
Chemical structures of PTP inactivators.
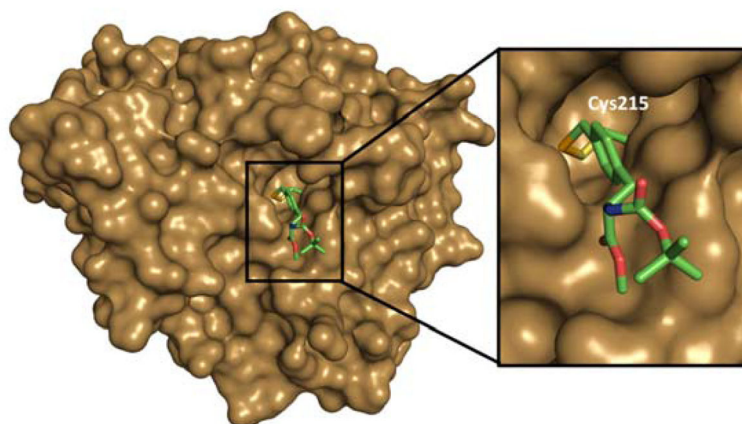
Fig. 23.
X-ray crystal structure of 50 complexed with PTP1B.[158] The figure was made using PyMol.
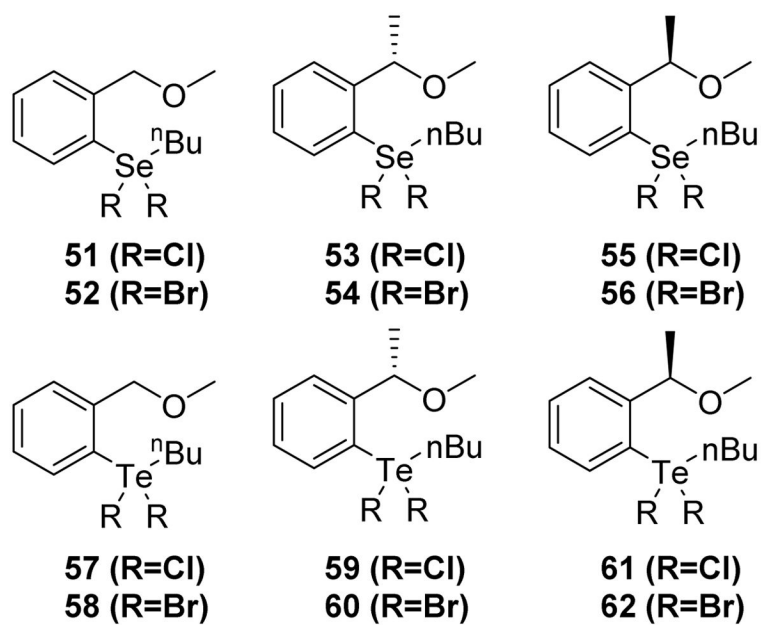
Fig. 24.
The chemical structures of organoselenanes and organotelluranes.[160]
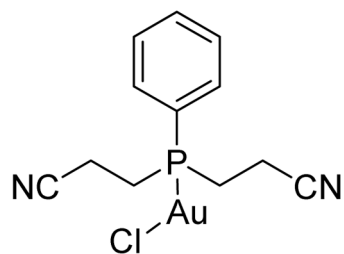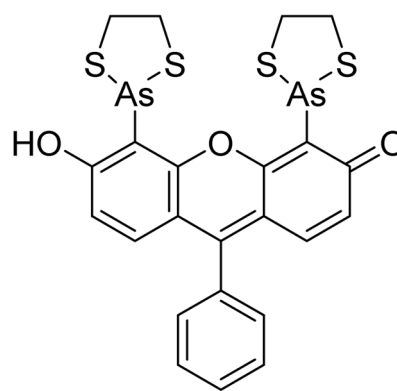
63   **FlAsH**

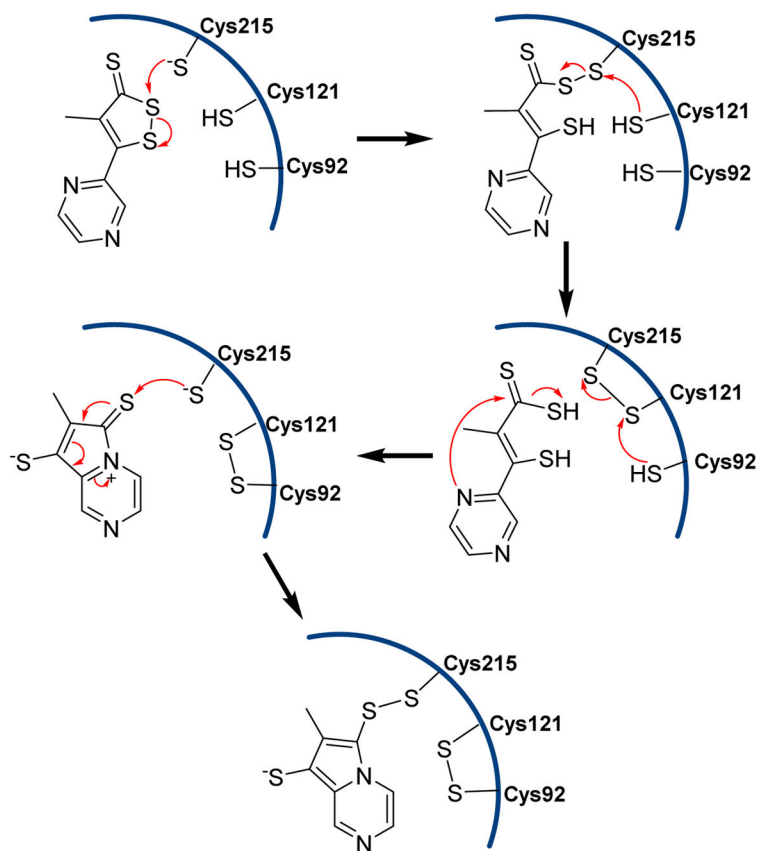**Fig. 25.**
Chemical structures of Au(I) complex and FlAsH.

Fig. 26.
The proposed mechanism of inactivation of PTP1B by oltipraz.

A)

Sulfenic acid

B)

Sulfenylamide
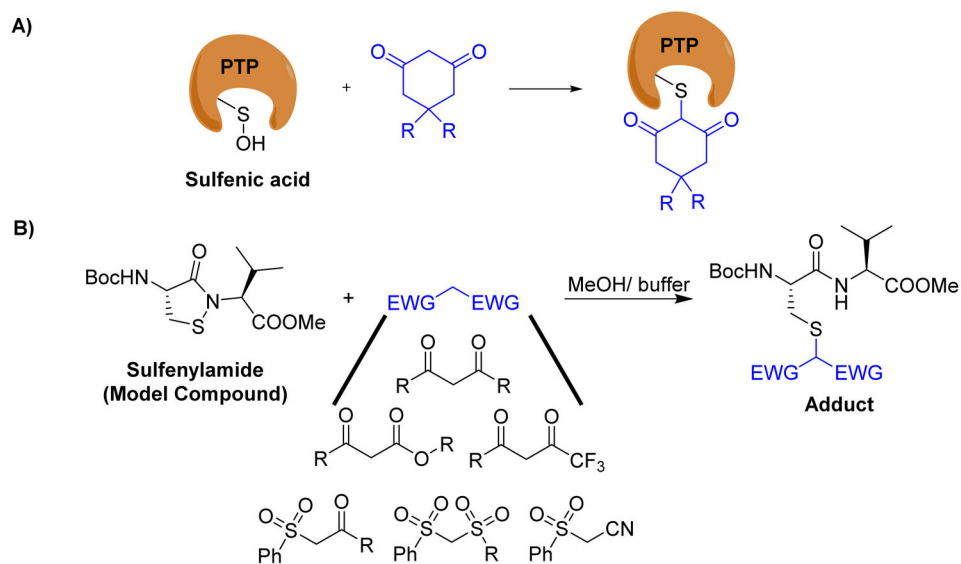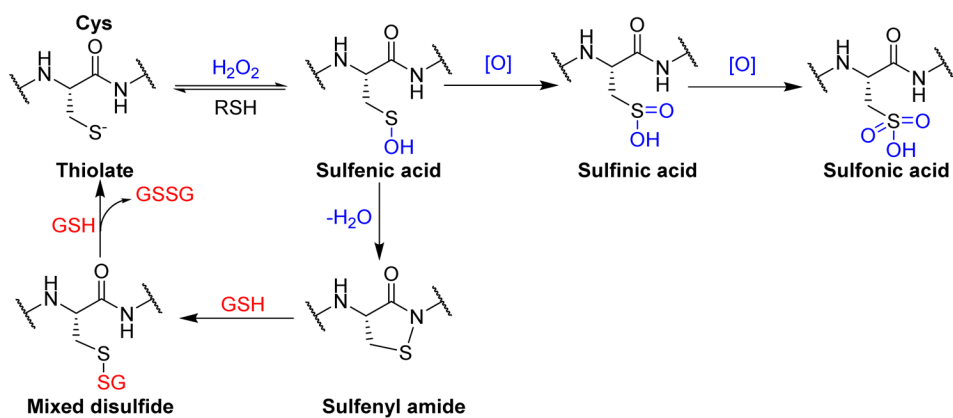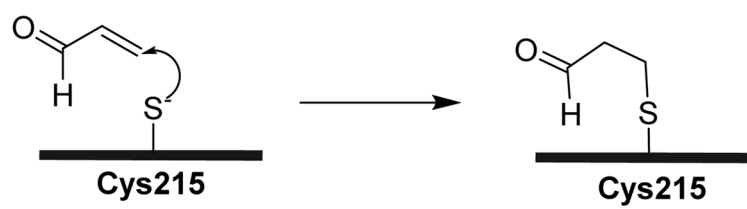(Model Compound)

MeOH/ buffer

Adduct

Fig. 27.
Covalent capture of oxidized forms of PTPs. A) Covalent modification of sulfenic acid by 1,3-diketone derivatives. B) Covalent capture of sulfenyl amide model compound by various carbon nucleophiles.

Scheme 1.
Redox regulation of protein tyrosine phosphatases.

Scheme 2.
The proposed mechanism of inactivation of PTP1B by acrolein.[122]

Table 1

Kinetic parameters reported for the inactivation of PTP1B and YopH by various S-nitrosothiols.[100]

Inhibitor	Enzyme	k_{inact} (min^{-1})	K_I (mM)	k_{inact}/K_I (M^{-1} min^{-1})
GSNO	PTP1B	0.046	0.466	99
	YopH	0.038	0.573	66
SNAP	PTP1B	0.109	0.969	113
	YopH	2.09	3.41	613
S-NO captopril	PTP1B	0.108	1.040	104
	YopH	0.124	0.848	146
Glucose-SNAP-2	PTP1B	0.111	3.014	37
	YopH	0.084	1.152	73