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## Inhibitors of Serine/Threonine Protein Phosphatases: Biochemical and Structural Studies Provide Insight for Further Development

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### Abstract

**Background:** The reversible phosphorylation of proteins regulates many key functions in eukaryotic cells. Phosphorylation is catalyzed by protein kinases, with the majority of phosphorylation occurring on side chains of serine and threonine residues. The phosphomonoesters generated by protein kinases are hydrolyzed by protein phosphatases. In the absence of a phosphatase the half-time for the hydrolysis of alkyl phosphate dianions at 25° C is over 1 trillion years;  $k_{\text{non}} \sim 2 \times 10^{-20} \text{ sec}^{-1}$ . Therefore, ser/thr phosphatases are critical for processes controlled by reversible phosphorylation.

**Methods:** This review is based on a search of the literature in available databases. We compare the catalytic mechanism of PPP-family phosphatases (PPPses) and the interactions of inhibitors that target these enzymes.

**Results:** PPPases are metal-dependent hydrolases that enhance the rate of hydrolysis ( $(k_{\text{cat}}/k_{\text{M}})/k_{\text{non}}$ ) by a factor of  $\sim 10^{21}$ , placing them among the most powerful known catalysts on earth. Biochemical and structural studies indicate the remarkable catalytic proficiencies of PPPases are achieved by 10 conserved amino acids, DXH(X)<sub>~26</sub>DXXDR(X)<sub>~20-26</sub>NH(X)<sub>~50</sub>H(X)<sub>~25-45</sub>R(X)<sub>~30-40</sub>H. Six act as metal-coordinating residues. Four position and orient the substrate phosphate. Together, two metal ions and the 10 catalytic residues position the phosphoryl group and an activated bridging water/hydroxide nucleophile for inline attack upon the substrate phosphorous atom. The PPPases are conserved among species, and many structurally diverse natural toxins co-evolved to target these enzymes.

**Conclusion.**—Although the catalytic site is conserved, opportunities for the development of selective inhibitors of this important group of metalloenzymes exist.

### Keywords

Phosphatase; inhibitor; okadaic acid; fostriecin; cantharidin; tautomycin; microcystin crystal structures

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## 1. INTRODUCTION

Reversible phosphorylation of structural and regulatory proteins plays a crucial role in the regulation of many cellular functions in eukaryotes. Protein phosphorylation occurs principally upon serine, threonine, and tyrosine residues and is mediated by members of an extremely large group of ser/thr- and tyr-protein kinases. Protein kinases catalyze the transfer of a gamma phosphate from adenosine triphosphate (ATP) to a side chain hydroxyl. There are >500 genes in humans known to encode protein kinases [1], with the number of potential gene products being much greater due to alternative splicing [2,3]. Phosphorylation affects an extremely large and diverse panoply of cellular processes including: cell growth and proliferation, gene transcription, transcript processing, translation, protein-protein interactions, subcellular localization, programmed cell death, turnover of cellular proteins and organelles, metabolism, muscle contraction, and cytoskeleton assembly and function [4-11]. Most kinases target substrates in a sequence specific manner [12-14].

The removal of phosphate is accomplished by one or more of the >150 ser/thr-, tyr-, or dual-specificity phosphatases, which catalyze the hydrolysis of seryl, threonyl, and/or tyrosyl phosphomonoesters [8,15-18]. The biological activity of a protein, its location within a cell, its stability, and interactions with other macromolecules can all be affected by its phosphorylation state, which is governed dynamically via coordinated regulation of the kinases and phosphatases that utilize it as a substrate.

Most tyrosine-specific and dual-specificity protein phosphatases share a common catalytic mechanism in which a covalent thiophosphate intermediate forms with the side chain of a catalytic cysteine (one family instead utilizes an aspartyl phosphate intermediate) [17,19-21]. In contrast, serine/threonine-specific protein phosphatases are metalloenzymes that utilize metal-ligated water/hydroxide as a nucleophile in the phosphomonoester hydrolysis reaction. Modern nomenclature divides the ser/thr phosphatases into two genetically and structurally distinct lineages, the PPP family and the PPM family. This review will focus on the biochemistry and structural biology of the PPP family phosphatases (PPPs) in mammals, discussing the common catalytic mechanism, interactions with natural toxins and small molecule inhibitors, and prospects for developing more selective inhibitors.

### 1.1. Serine/Threonine Protein Phosphatases:

Deciphering the PPP-family literature can be bewildering, especially to a novice to the field. Therefore, it should be useful to start with a short discussion of the evolution of the phosphatase nomenclature. Historically, both protein kinases and protein phosphatases were first named by the substrates they acted upon. The kinase that phosphorylates phosphorylase was named phosphorylase kinase, and phosphorylase phosphatase seemed to be an appropriate name for the phosphatase that catalyzed dephosphorylation [22]. However, later it became apparent that, *in vitro*, more than one phosphatase could catalyze the same reaction, and many phosphatases demonstrate little substrate specificity. To establish order in the field, Ingebritsen and Cohen in the 1980s proposed a system of nomenclature that is currently obsolete, but the names are still pertinent because their “traditional” nomenclature lives on and is still in common usage within the field [23].

The traditional nomenclature was based upon the notion that the ser/thr phosphatase activities identified and characterized in the literature up to that time were the result of only four principal enzymes with phosphatase activity: type 1 (PP1), type 2A (PP2A), type 2B (PP2B), and type 2C (PP2C). This classification was based upon biochemical characteristics, sensitivity to endogenous inhibitory proteins, and the limited substrate specificity that could be demonstrated *in vitro*. In the 1990's we learned that PP1, PP2A, and PP2B are highly conserved across taxa and are closely related to each other, sharing ~40% sequence identity within the ~250 residue conserved core domain. Genomic analysis would also reveal separate genes encoding isoforms of PP1 ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), PP2A ( $\alpha$ ,  $\beta$ ) and PP2B ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). However, the lack of substrate discriminatory power raised doubts about the ability of these enzymes to meaningfully participate in complex signaling pathways, as highly specific interactions between components are necessary to minimize interference and unregulated cross-talk between different pathways, which is needed to ensure high-fidelity transmission. Therefore, for years many erroneously viewed ser/thr protein phosphatases as simple "house keeping" enzymes.

Advances in DNA sequencing technologies allowed the entire genome of many organisms to be sequenced in their entirety, and today we know that PP1, PP2A and PP2B and their isoforms are actually catalytic subunits that are shared by many different holoenzymes. The current nomenclature is based on amino acid similarity and conservation of catalytic mechanisms. This classification system divides phospho-ser/thr phosphatases into two major families (PPP and PPM). PPP has an extra P because the PP designation was given to protein proteases. In humans there are also eight CTD aspartate-based phosphatases that have a different catalytic mechanism and will not be addressed further in this review.

PP1, PP2A, and PP2B share a common catalytic mechanism and represent the "founding" members of the PPP-family. Novel mammalian PPP phosphatases include PP4, PP5, PP6, and the two isoforms of PP7/PPEF: PPPEF1 and PPPEF2 [8,16,24,25]. The primary amino acid sequence of PP2C shares no sequence similarity to the PPP family and was placed as a member of the separate and distinct PPM-family [16]. The reclassification is borne out by the crystal structures of PPP and PPM phosphatases produced from the 1990s onward, which reveal that PPP-family members show great structural similarity to one another and possess a fold distinct from the PPM family.

Based upon sequence comparisons, the mammalian PPP phosphatases and their isoforms can be further placed into four basic groups or subfamilies [16,18,26]: PP1, PP2A-like (which includes PP4 and PP6), PP2B, and PP5-like (which includes PP7/PPEF; Figure 1).

Examples of each of the four subfamilies are found in yeast as well as mammals, strongly suggesting the entrenchment of all four subfamilies across the entirety of the Eukarya. Similar to PP1 and PP2A, PP4 and PP6 serve as the catalytic subunits of PPP-holoenzymes. Therefore, the name of the subunit with the catalytic site was designated with the letter C (e.g. PP1 is now PP1C/*PPP1C* with the gene often designated in *italic* lettering. A confusing point in the literature is that, when it was realized that PP2A acted as a common catalytic subunit shared among many PPP2-holoenzymes, PP2A was designated as PP2AC or PP2Ac to indicate which subunit of a holoenzyme was being discussed. However, in the

current system PP2A/PP2AC is now PPP2CA or PPP2CB, with A and B replacing the  $\alpha$   $\beta$  designation for the PP2AC isoforms. PP2B officially became PPP3C, but in the literature it is often referred to as PP2BC or calcineurin, which was the first name given to this protein. Nomenclature issues aside, all PPP-family enzymes display a great deal of sequence similarity within the central conserved core domain (associated with the catalytic activity) but their N- and C- termini are divergent. This is particularly striking for PPP3C, PPP5C, and PPP7C, which have large N- or C- terminal extensions forming accessory domains that regulate catalytic activity and/or protein-protein interactions.

Although many of the PPP phosphatases display little substrate specificity *in vitro*, it is now apparent that diversity of function and discriminatory power in the PPP phosphatases is primarily due to the existence of numerous distinct accessory domains having targeting and/or regulatory functions, which act to adapt the catalytic subunits to particular physiological roles by controlling sub-cellular localization, catalytic activity, and/or substrate specificity [8,24,27,28]. In some cases (e.g. PPP5C and PPP7/EF), the catalytic domain has been fused directly to such an accessory domain [25,29,30]. More typically, however, the accessory domains are provided by separate polypeptide subunits that form multimeric holoenzymes [24,27,28,31-34]. In this way, a small number of evolutionally conserved catalytic subunits are able to assemble into a diverse array of functionally specific holoenzymes. This stands in stark contrast to the serine/threonine protein kinases, where functional diversity and specificity of interaction appear to have evolved via gene duplication and subsequent divergence that allowed the florescence of a vast multiplicity of different catalytic isoforms [1,35-37].

Although it is beyond the scope of this review to address the assembly all PPP-family holoenzymes, we will provide a few examples for each family to address holoenzyme differences and to introduce the nomenclature of the regulatory and scaffold proteins.

**1.1.1. PPP1C:** PPP1 type phosphatases are involved in regulation of actions as diverse as glycogen metabolism, synaptic plasticity, cell cycle progression, RNA splicing, and smooth muscle contraction [7,8,27,28,38]. Isoforms of the PP1 catalytic subunit are expressed from three genes in humans: PPP1CA, PPP1CB and PPP1CC that encode PP1 $\alpha$ , PP1 $\beta/\delta$  and two PP1 $\gamma$  isoforms, respectively. Transcripts from PPP1CC undergo tissue-specific alternative splicing to produce either the ubiquitously expressed PPP1C $\gamma$ 1 or the testis-specific PPP1C $\gamma$ 2 variant [39-41]. Overall, the PPP1C isoforms are very closely related (>90% identity) the main differences being near the N- and C-termini. Unlike their protein kinase counterparts, PPP1 catalytic subunits exhibit little substrate selectivity and lack any obvious consensus sequence for phospho-site recognition. In the cellular context, protein phosphatase 1 enzymes exist as multi-subunit holoenzyme complexes composed of a highly conserved catalytic subunit in complex with one or two variable regulatory subunits. A very large number of regulatory subunits have been identified so far [27,33,34], including proteins involved in subcellular targeting of the holoenzyme complex, modifying substrate specificity, or inhibiting catalytic function. Mixing and matching catalytic subunits with such a large variety of accessory proteins allows for an incredibly diverse array of functionally distinct PP1 holoenzymes to be assembled from a rather meager set of catalytic subunit gene products.

**1.1.2. PP2A/PPP2CA/PPP2CB:** Like PP1, PP2A type catalytic subunits lack an obvious consensus sequence and can dephosphorylate a variety of substrates *in vitro* and *in vivo*. Two isoforms,  $\alpha$  and  $\beta$ , are expressed in human cells from two genes; *PPP2CA* and *PPP2CB*, respectively. PP2A enzymes function *in vivo* principally as heterocomplexes, each consisting of a PP2A catalytic subunit (C subunit), one of two A-type subunits ( $\alpha$  or  $\beta$ ; encoded by *PPP2R1A* and *PPP2R1B*) and one of ~18 or more regulatory B-type subunits from four distinct families (B, B', B'', and B''') expressed from ~14 genes [24,28,32,42] (Figure 2). The A subunit acts as a molecular scaffold allowing close association of the catalytic subunit with a B subunit, and specific roles for most of the many possible holoenzyme complexes have yet to be clearly elucidated. However, PPP2AC containing complexes participate in many signal transduction pathways involved in regulation of the cell cycle, cell size, migration, metabolism, and apoptosis [32,42-47].

**1.1.3. PP2B/calcineurin/PPP3C:** The PP2B type phosphatases (also referred to as calcineurin) play a major role in  $\text{Ca}^{2+}$ -dependent signaling pathways in eukaryotes. Three isoforms of the catalytic subunit are expressed from three genes (*PPP3CA*, *PPP3CB*, *PPP3CC*) in humans. In contrast to PPP1 and PPP2, PP2B demonstrates relatively narrow substrate specificity, efficiently dephosphorylating inhibitor 1 and the RII regulatory subunit of cyclic AMP dependent protein kinase (PKA). PP2B/PPP3C holoenzymes exist as heterodimers comprised of a 58-64 kDa A subunit contains the catalytic and calmodulin-binding domains and a 19 kDa  $\text{Ca}^{2+}$ -binding regulatory subunit (calcineurin B). The phosphatase activity of PP2B/PPP3C is regulated by two structurally similar calcium binding proteins (calmodulin and calcineurin B). Under conditions of low intracellular  $\text{Ca}^{2+}$  concentration ( $<10^{-7}$  M) calcineurin B is bound to PP2B and the A-C dimer is inactive, with the calmodulin binding domain situated in the active site cleft blocking substrate access. At high intracellular calcium concentration,  $\text{Ca}^{2+}$  binding to calmodulin causes conformational changes enabling binding to PP2B resulting in abrogation of autoinhibition. For reviews of PP2B/calcineurin activity see Rusnak and Mertz [48], Musson and Smit [49], and Nygren and Scott [50].

**1.1.4. PP4/PPP4C:** PPP4C has diverse functions in the cell, including spliceosome assembly, regulation of microtubule growth and nucleation, growth and maturation of the centrosome during cell division, cell migration, insulin signaling and regulation of RNA polymerase III [51-55]. The PPP4 catalytic subunit has only one isoform that is expressed from a single gene (*PPP4C*). PPP4C is highly homologous to PPP2CA/PPP2CB, sharing ~65% identity at the level of their primary amino acid sequences. Like PPP1 and PPP2, functional PPP4 enzymes *in vivo* consist of multimeric holoenzyme complexes formed from a catalytic subunit combined with a variety of regulatory subunits. There are five genes encoding the principal PPP4 regulatory subunits: *PPP4R1*, *PPP4R2*, *PPP4R3A*, *PPP4R3B*, and *PPP4R4* [51,56-58]. The roles of individual PPP4-holoenzymes are poorly understood.

**1.1.5. PP5/PPP5C:** PPP5C is involved in regulation of many stress- and hormone-induced signaling cascades and can act as a negative regulator of apoptosis. PPP5C forms part of the hsp90 chaperone machinery, regulating the phosphorylation states of chaperone complexes and is involved in heat shock protein 90 (HSP90) client maturation [59-67]. PPP5

is expressed from a single gene (PPP5C in humans) and contains a catalytic domain sharing 42-43% sequence identity with PPP1C/PPP2CA/PPP2CB /PPP3C. However, PPP5C has a unique N-terminal domain that both regulates catalytic activity and mediates interactions with binding partners. Structural studies indicate that the N-terminal domain of PPP5C is connected to the catalytic domain by a flexible linker/tether sequence that allows a tripartite tetratricopeptide-repeat (TPR) motif within the N-terminal domain to adopt a conformation that occludes the active site via the formation of stabilizing interactions with the catalytic domain and an adjacent C-terminal J-helix [68,69]. Rather than forming stable heterocomplexes with myriad regulatory subunits, PPP5's TPR-domains are involved in the binding of PPP5C to "TPR-docking" sites on proteins such as heat shock protein 90, the best characterized interaction partner to date. When PPP5 is displaced from its binding partners, the N-terminal/C-terminal interaction is inhibitory. When in a complex with other proteins, a binding-induced conformational change opens the active site to substrates, thereby "activating" PPP5C. Removal of the autoinhibitory N-terminal domain by proteolysis results in a bare catalytic domain with greatly increased activity [70,71]. PPP5C is also activated by the addition of micellar concentrations of unsaturated fatty acids (e.g. arachidonic acid, palmitate) or micromolar concentrations of fatty acid CoA esters, which may act by ablating the auto-inhibitory function of the N-terminal domain [70,72,73].

**1.1.6. PP6/PPP6C:** PPP6C also has diverse functions in the cell, including the regulation of mitotic progression, meiosis II exit, NF- $\kappa$ B signaling, autophagy, and T cell development. The disruption of PPP6 expression has also been linked as a driver mutation in the development of melanoma [74-80]. Like PPP4C, there is only one isoform of the catalytic subunit (PPP6C) that is highly similar to PPP2CA/B, sharing >50% identity. Like PPP2, PPP6 functions as a trimeric holoenzyme that consist of PPP6C, one of three regulatory proteins (PPP6R1, PPP6R2, PPP6R3), and one of three ankrin repeat domain containing scaffolding proteins (ANR28, ANR44 and ANR52) [81,82]. The combinatorial assembly results in nine distinct PPP6C holoenzymes, and the functions of the individual holoenzymes are poorly characterized.

**1.1.7. PP7/PPEF1/2:** PP7 type phosphatase, like PP2B/PPP3C, is regulated by calcium [8,25]. In humans there are two highly homologous isoforms (PP7C/EF1 and PPEF2) expressed from two genes (*PPP7C/PPPEF1* and *PPPEF2*, respectively). PPP7 expression is restricted to primary sensory neurons, and it has unique N- and C-terminal regions. The N-terminal domain contains a calmodulin binding site, while the extended C-terminal domain contains five EF-hand motifs and has been implicated in activation of catalytic activity by calcium [25,83]. Human PPP7 shares homology with *Drosophila* retinal degeneration C Gene product (rdgC). However, to date, the physiological roles for PPP7C have remained elusive.

## 1.2. Architecture of the Phosphatase Domain:

Several excellent reviews discuss the structural biology of PP1, PP2A, and PP2B holoenzyme complexes and their function/assembly [48,49,84-86]. However, the details of subunit interactions within PPPase holoenzyme complexes is beyond the scope of this review, which will focus upon the catalytic mechanism and structures of the conserved

phosphatase domain with a view to inform efforts to develop type-specific inhibitors of these enzymes.

PPP family phosphatases share a conserved catalytic core domain of ~250 residues (Figure 3). Catalytic subunits of PPP1C, PPP2C, PPP4C, and PPP6C are of roughly similar size and display high homology within this core domain, differing from one another mainly within short non-conserved N- and C-terminal sequences. Homology is particularly high (~60-65% identity) between PPP2C, PPP4C, and PPP6C. PP2B/PPP3C departs from this trend by having a long non-conserved C-terminal extension containing regulatory domains (e.g. a calmodulin binding domain) as well as two small stretches of non-conserved sequence within the otherwise conserved core that are inserted into the  $\beta$ 7- $\beta$ 8 and  $\beta$ 11- $\beta$ 12 loops, respectively, which are positioned far from both the catalytic center and the regulatory B subunit binding interface. PPP5C differs from other members of the family in that it possesses a large N-terminal regulatory/targeting domain, and a unique C-terminal alpha helix. PPP7C isoforms have a ~40 residue non-conserved insert in the catalytic core and also diverge in the C-terminal region, which contains a calcium-binding regulatory domain with EF hand motifs. To date, crystal structures have been reported for PPP5C and the catalytic subunits PPP1CA (PP1 $\alpha$ ), PPP1CC (PP1 $\gamma$ <sub>1</sub>), PPP2CA (PP2A $\alpha$ ), PPP3CA (PP2B $\alpha$ /calcineurin) and PPP3CB (PP2B $\beta$ /calcineurin). As the catalytic domain (residues 169-499) of PPP5C has yielded many of the highest resolution structures from this family [68,87,88], the overall architecture of the phosphatase fold and the basic catalytic mechanism will be discussed here primarily with reference to PPP5C.

The PPP phosphatase fold, as exemplified by the PPP5- catalytic domain [68,69,87,88], has a compact  $\alpha/\beta$  fold comprised of 11  $\alpha$ -helices and 14  $\beta$ -strands (Figure 4). Eleven of 14 strands ( $\beta$ 1- $\beta$ 6 and  $\beta$ 10- $\beta$ 14) form two  $\beta$ -sheets that are arranged in a  $\beta$ -sandwich structure in the center of the molecule. One side of this  $\beta$ -sandwich is occupied by three short helices ( $\alpha$ G- $\alpha$ I) and a three-stranded  $\beta$ -sheet ( $\beta$ 7- $\beta$ 9) that nearly forms a separate subdomain. On the other side of this  $\beta$ -sandwich there is an  $\alpha$ -helical bundle that includes six helices ( $\alpha$ A- $\alpha$ F) plus the A'-helix, which is immediately C-terminal to a non-conserved TPR linker sequence. The first helix is designated A' because, in spite of very low sequence homology within the PPP family in this region, significant structural similarity still exists. PPP5C also possesses a non-conserved C-terminal regulatory subdomain composed of the J-helix tethered by a short linker extending from the end of the  $\beta$ 14-strand.

### 1.3. Catalytic Mechanism:

For ease of discussion, in this section the catalytic mechanism is discussed using the amino acid sequence numbering from PPP5C. Much work has established that alkyl phosphate dianions, which are found in phosphorylated serine or threonine residues, are markedly stable. The half-time for hydrolysis of such groups at 25°C is estimated to be over 1 trillion years;  $k_{\text{non}} \sim 2 \times 10^{-20} \text{ sec}^{-1}$  [89]. Substrate turnover rates ( $k_{\text{cat}}$ ) for PPPases typically range from ~1 to ~100  $\text{sec}^{-1}$ , implying a profound  $\sim 10^{21}$ -fold rate enhancement. These enzymes are therefore among the most powerful known catalysts, with catalytic proficiencies ( $[k_{\text{cat}}/\text{kM}]/k_{\text{non}}$ ) of  $\sim 10^{25}$ - $10^{26} \text{ M}^{-1}$ . [89-92] rate enhancements require not

only increasing the nucleophilicity of water but also an exquisite alignment of the attacking nucleophile with bound substrate as well as profound stabilization of the transition state.

The catalytic center is dominated by two closely apposed metal ions that are tightly bound to the enzyme through interactions with multiple active site residues. Most of the existing crystal structures of PPPases have been derived from catalytically active recombinant proteins that were heterologously expressed in the presence of  $Mn^{2+}$ . X-ray fluorescence of recombinant PPP5C crystals revealed the presence of Fe, Zn, and Mn [68], proton-induced x-ray emission analysis showed Fe and Mn were present in recombinant PPP1CA crystals [93], and ICP atomic emission spectroscopy of recombinant PPP2CA expressed in insect cells identified bound metal as Mn [94]. However, metal analyses of native PPPase enzymes purified from mammalian tissues/cells have consistently shown a combination of Fe and Zn bound to the catalytic subunits [95-98]. Electron paramagnetic resonance spectroscopy studies strongly suggest an  $Fe^{2+}/Zn^{2+}$  binuclear site in native PP2B/PPP3C [96] and, by extension, this is likely to also be the case for the rest of the family. Even though a Fe/Zn bimetal site seems to exist natively, enzyme activity appears to be tolerant of substitution at these sites by manganese.

The bimetal site is located at the base of a shallow depression on the PPPase surface formed by the residues of four loops;  $\beta$ 4- $\alpha$ D,  $\alpha$ G- $\alpha$ H,  $\beta$ 10- $\beta$ 11, and  $\beta$ 12- $\beta$ 13 (Figure 4). A structural comparison of eukaryotic PPPases reveals an active site motif consisting of 10 conserved amino acid residues; Asp242, His244, Asp271, Asp274, Arg275, Asn303, His304, His352, Arg400, and His427 (PPP5C numbering; Figure 5).

As both enzyme kinetics [99] and kinetic isotope effect studies [100] of phosphomonoester hydrolysis by PP2B indicate that the substrate dianion is the catalytically active species, the bound phosphate (primarily dianionic at the crystallization pH) offers a reasonable structural analog of the substrate phosphoryl moiety. The structure of the PPP5C catalytic domain with bound phosphate (PDB code 1S95) thus gives a model of substrate-enzyme interactions in a near attack configuration. Six of the 10 conserved residues (Asp242, His244, Asp271, Asn303, His352, and His427) are metal-coordinating and four (Arg275, Asn303, His304, and Arg400) are observed to be directly involved in substrate binding via strong hydrogen bonds to the phosphoryl oxygens [68] (Figure 6). Mutation of either equivalent arginine in PPP1C or PP2B/PPP3C adversely affects substrate binding and catalysis and mutation of Asp274 or His304 equivalent residues severely impacts catalytic activity [90,101]. In the substrate analog complex, His304 forms a short, strong hydrogen bond (2.6 Å) with  $O^4$  of phosphate (equivalent to the substrate leaving group oxygen) and is well positioned to act as a general acid in the hydrolysis reaction while Asp274 hydrogen bonds with the His304 imidazole and is thought to increase the imidazole pKa, stabilizing the histidine side chain in a cationic state that is capable of protonating the leaving group [68]. Alternatively, even without a general acid function, positively charged His304, along with Arg275 and Arg400, could contribute to electrostatic stabilization of the transition state by neutralization of the negative charge developing on the leaving group oxygen.

In early work on the enzymology of PP2B, Martin et al [102] proposed three possible types of catalytic mechanisms. The first required nucleophilic attack by an active site residue to



form a phosphoenzyme intermediate. This type of mechanism occurs in some families of protein phosphatases (e.g. protein tyrosine phosphatases) but attempts to detect evidence of such intermediates in PPPases [99,102,103] have failed, thereby eliminating this mechanism from consideration. Another possibility considered was the unimolecular breakdown of monoanionic substrate ligated to active site metal ions. However, subsequent kinetic isotope effect data indicated that PPPases utilize dianions, rendering this mechanism unlikely [100]. Finally we are left with the most viable type of mechanism considered, that of nucleophilic attack by a metal-coordinated water (or hydroxide) upon the tetrahedral ground state phosphorus [68]. Water-derived species generally display nucleophilicities in the following order:  $\text{H}_3\text{O}^+ \ll \text{metal-ligated water} < \text{unligated water} \ll \text{metal-ligated hydroxide} < \text{unligated hydroxide}$  [104,105]. Coordination of water to a metal ion greatly reduces its pKa, thus the catalytic effect here derives from the generation of local concentrations of the nucleophile far in excess of what would otherwise be possible at a given pH rather than direct activation of the nucleophile.

Returning to the substrate analog complex (1S95), we see that phosphate is coordinated to the active site metal ions in a bidentate fashion (Figure 6).

This may contribute to rate enhancement through stabilization of negative charges building up on the phosphoryl oxygens in the transition state and may also make the phosphorus more electrophilic by polarizing the P-O bonds in the ground state. Also coordinating the metals are two waters/hydroxides that comprise the most likely candidates for the catalytic nucleophile. One of these is terminally ligated while the other sits at a bridging position between the two metals. While bridging two Lewis-acidic metal centers may reduce the nucleophilicity of a bound hydroxide below that of the corresponding terminally-ligated species, the pKa of water at the bridging position would be expected to be much lower than at the terminal position. Thus, at the approximately neutral pH optima of PPPases, the bridging position is likely to produce a much greater population of reactive hydroxide nucleophiles. This suggests the bridging hydroxide is the more likely catalytic nucleophile. Geometric considerations also argue for this [68]. The bridging hydroxide in 1S95 is very close to the phosphate phosphorus (3.0 Å, less than the van der Waals contact distance) and is directly in line (i.e. a nearly 180° angle) with phosphorus and O<sup>4</sup> (equivalent to the leaving group oxygen). Additionally, there is a short hydrogen bond between the bridging hydroxide and the backbone carbonyl of His427 with a ~109° angle between the carbonyl O, hydroxide O, and P. This appears to be an example of orbital steering [106], with the metals and His427 acting to fix the orientation of a lone pair of electrons from the hydroxide toward the substrate phosphorus atom. The likely reaction mechanism, derived from available data, is shown schematically in Figure 7.

The bridging hydroxide [1] is precisely positioned for in-line nucleophilic attack upon the phosphorus of incoming substrate by the active site metals and carbonyl oxygen of His427. Kinetic isotope effects measured for pNPP hydrolysis by PP2B [100] and the structurally related bacteriophage lambda phosphatase [107] indicate a highly dissociative, metaphosphate-like transition state (as is also the case for the uncatalyzed reaction), with advanced bond dissociation to the leaving group and little bond formation to the attacking nucleophile. Since the attacking nucleophile and the leaving group oxygen are held in

place by multiple strong interactions with the enzyme, neither are likely to move relative to one another during the evolution of the transition state. As the reaction progresses from step [2] to [3], the phosphorus moves along the reaction coordinate toward the bridging hydroxide, while the angles between the equatorial P-O bonds increase, forming a planar metaphosphate-like dissociative transition state [3] with the attacking nucleophile and leaving group oxygen sitting at axial positions with low bond order to P. In the transition state the equatorial P-O bonds have little double-bond character and are highly polarized. These polarized bonds are stabilized through interactions with Arg275, Asn303, and Arg400 as well as the active site metal ions. The negative charge developing on the leaving group oxygen as that P-O bond dissociates is stabilized through interactions with Arg275 and His304. Strong electrostatic effects produced by the dinuclear metal center underlie a major part of its catalytic role. By stabilizing negative charges, the metals not only promote deprotonation of the bridging water but can also make the phosphoryl moiety more susceptible to attack by stabilizing the polarized P-O bonds that develop during the reaction. In a similar way, Arg275 and Arg400 are not only necessary for binding and orienting the substrate, but also provide stabilizing interactions for the transition state.

As the reaction continues past step [3] toward step [4], the phosphoryl group continues its inversion about phosphorus. Bond order to the attacking nucleophile increases and bond dissociation to the leaving group nears completion. At some point during bond dissociation, the proton affinity of the leaving group oxygen increases enough to accept a proton from His304, which acts as a general acid. At completion [4], the incoming hydroxide nucleophile has become a hydroxyl group of the phosphate dianion product bound to the dinuclear metal center in an unstable tridentate mode and is subsequently expelled while the bridging coordinating position is repopulated from bulk solvent. The high solvent accessibility of the shallow active site combined with the relatively low turnover (*k<sub>cat</sub>*) of phosphoprotein/phosphopeptide substrates by PPPases [89] allows enough time and opportunity during the catalytic cycle for regeneration of the active ionization state (i.e. bridging hydroxide nucleophile and protonated His304 general acid) to take place through simple exchange of protons with intracellular bulk solvent and/or buffer molecules (e.g. organic acids, phosphates, and free amino acids) since proton exchange reactions can proceed at rates far greater than typical PPPase *k<sub>cat</sub>*'s [108].

#### 1.4. Inhibitors of PPP family phosphatases:

Going back in history ~25 years, the dogma of the time was that protein kinases were not suitable targets for drug development. Fortunately, not everyone followed the dogma, and today after an extensive effort there are > 20 drugs targeting protein kinases (i.e. gefitinib, dabrafenib, STI-571/Gleevec, ceritinib) that are used in a clinical setting to treat human disease, most for the medical management of human cancers. Currently, there are many that argue the PPPases are not valid drug targets. However, like the view of PPPases as simple house keeping enzymes, this opinion is slowly falling out of favor. Still, developing PPPase inhibitors into drugs will be challenging because as discussed above the PPP-family phosphatases share a catalytic mechanism, and many family members retain a high degree of structural similarity in the regions surrounding the active site. In addition, as will

be discussed in more detail below, high affinity non-selective inhibitors of PPP-catalytic activity are highly toxic to most, if not all, human cells.

**1.4.1. Immunophilin ligands:** Proof that PPP-family phosphatases can be targeted for drug development already exist, in the respect that the cyclic undecapeptide cyclosporin A and the macrolide tacrolimus (FK506) have already been developed into highly effective immunosuppressant drugs. Both are potent and selective inhibitors of PP2B/PPP3C holoenzymes *in vivo* [109]. However, their mode of action is not typical in that binding to an accessory protein (an immunophilin) is first necessary in order to make the drug competent for phosphatase inhibition. Immunophilins are highly conserved chaperone proteins typically having peptidylprolyl isomerase activity [110-112]. The two main families are the cyclosporin A binding cyclophilins (Cyp) and the FK506 binding proteins (FKBP), which bind macrolides such as rapamycin and tacrolimus (FK506). The binary complex of CypA with cyclosporin A or FKBP-12 with tacrolimus is able to dock to and inhibit the PP2B holoenzyme (catalytic and regulatory subunits; Figure 8). Inhibition of PP2B by these immunophilin/immunosuppressant complexes is extremely selective due to extensive interactions between the inhibitor complex and a composite surface formed by the non-conserved C-terminal helix of the PP2B catalytic subunit and the regulatory B subunit, which is not shared by other PPP-ases [48,49,112-116].

**1.4.2. Natural compounds targeting PPPases.**—PPPases are evolutionarily-primitive enzymes that emerged with the development of *Archaea*. During evolution many structurally diverse natural compounds targeting these enzymes evolved, likely as a means of chemical defense to prevent predation. The first natural toxin shown to act as a PPPase inhibitor [117] was okadaic acid, a complex polyether produced by marine dinoflagellates (e.g *Prorocentrum sp.* and *Dinophysis sp.*). Microcystin-LR, a cyclic heptapeptide produced by fresh water cyanobacteria (*Microcystis sp.*) and nodularin, a structurally related compound produced by marine cyanobacteria (i.e. *Nodularia sp.*), were shown to inhibit the same phosphatases (PPP1C and PPP2) a few years later [118,119]. Analysis of extracts from red algae and marine sponges identified thyrseferyl 23-acetate, calyculins and dragmacidins [120-123]. Extracts from *Streptomyces sp.* yielded fostriecin, phostriecin, sultricin [124-126], cytostatin [127], phospholine [128], leustroducsins [129], phoslactomycins [130], tautomycin [131] and tautomycetin [132]. Extracts of ~1500 species of blister beetles contain cantharidin [133]. Although structurally diverse, (Figure 9), the aforementioned compounds all act by inhibiting a subset of the “toxin sensitive PPP-family members (PPP1C, PPP2CA/B, PPP4C, PPP5C and PPP6C) and the binding affinity and inhibitory activity of several differ substantially among the sensitive enzymes. To date, inhibitor-PPPase co-crystal structures have been reported for okadaic acid, microcystin-LR, nodularin, tautomycin, calyculin A, cyclosporine A, cantharidin, and analogues of cantharidin. Considerable insight into means of targeting PPPases can be gained from examining the structures that currently exist.

**1.4.3. Cyanobacterial toxins:** Microcystins are a family of toxic cyclic heptapeptides produced by certain cyanobacteria, with toxicity comparable to organophosphate nerve agents [134]. Microcystin was first identified as a highly toxic compound produced by

*Microcystis aeruginosa* called fast death factor [135,136]. In vivo, microcystins primarily target the liver, because transport into hepatocytes occurs actively, via specific organic anion transporting polypeptides (OATPs) [137]. Microcystin-LR (MCLR) was first reported to potently inhibit PPP1C and PPP2CA/B [118]. However, later it became clear that it also inhibits PPP4, PPP5, and PPP6, acting on all with sub- to low-nM IC<sub>50</sub> [138-140]. Now there are over 50 chemical variants of microcystin that act as potent, but rather non-selective inhibitors of these PPPases. PP2B is much less sensitive, having an IC<sub>50</sub> of ~200 nM [141], and PPP7 is very insensitive, being unaffected by 100 nM MCLR [25].

The crystal structure of the PPP1C $\alpha$ :MCLR complex was among the first structures of a PPPase catalytic domain to be reported [142]. A view of MCLR in its binding site is presented in Figure 10 (Top; PDB code 1FJM). MCLR interacts with three distinct regions on PPP1C $\alpha$ . First, it interacts both directly and indirectly with the highly conserved areas in and around the active site. Microcystin interacts with the active site metal ions by forming hydrogen bonds between two coordinated waters and the cyclic peptide glutamate side chain carboxyl and adjacent backbone carbonyl. It also interacts with Tyr134 and the absolutely conserved catalytic Arg96 via interactions with the D-erythro- $\beta$ -methylaspartate (Masp) moiety of the ligand. These interactions help position the cyclic peptide backbone directly over the catalytic center, blocking substrate access. Second, the Adda side chain of MCLR interacts with residues in the hydrophobic groove of PPP1C $\alpha$ , some of which are not conserved across the PPP family. The close packing of the Adda moiety suggests a significant contribution to binding affinity arises from desolvation/hydrophobic interactions. Third, the  $\beta$ 12- $\beta$ 13 loop adjacent to the active site engages in important interactions with MCLR side chains. Tyr272 (conserved across the family) of PPP1C $\alpha$  participates in a hydrophobic interaction with the cyclic peptide leucine side chain while the thiolate of Cys273, conserved only in the MCLR-sensitive PPPases (PPP1C, PPP2C, PPP4C, PPP5C, and PPP6C), is seen to react with the  $\alpha,\beta$ -unsaturated carbonyl of methyldehydroalanine (Mdha) to form a covalent adduct. Comparison with later structures of PPP1C $\alpha$  or PPP1C $\gamma$ <sub>1</sub> also show that the  $\beta$ 12- $\beta$ 13 loop shifts slightly in the MCLR complex to avoid a steric clash with the conserved Tyr272, suggesting that the flexibility of this loop may be important for high affinity binding [143].

The structure of the PPP2C:MCLR complex has also been reported [94] (Figure 10; bottom; PDB code 2IE3). Intermolecular interactions in this complex are, overall, quite similar to what is observed for PPP1C $\alpha$ :MCLR. The bound conformation of MCLR is virtually identical in both cases and is positioned similarly within the active site and hydrophobic groove. A major difference is observed in the interactions with the  $\beta$ 12- $\beta$ 13 loop, which has a small sequence segment that is not conserved with PPP1C. In the PPP2C:MCLR complex, the  $\beta$ 12- $\beta$ 13 loop adopts a different conformation and Cys269, present only in PPP2C, PPP4C, and PPP6C, forms the covalent adduct with the electrophilic methyldehydroalanine residue of the inhibitor. Other differences include interactions with non-conserved residues in the hydrophobic groove.

**1.4.5. Okadaic acid:** Okadaic acid (OA) and its close structural analogs, the dinophysistoxins (DTXs), are complex polyether fatty acids produced by several phytoplanktonic species and subject to bioaccumulation in filter feeders and their predators

[144,145]. OA was first isolated from the black sponge *Halichondria okadai* as one of the principal causative agents of diarrhetic shellfish poisoning (DSP). DSP is characterized by incapacitating diarrhea and vomiting. The symptoms are usually self-limiting without medical intervention and subside in 1-2 days. OA is membrane permeable, and the discovery that OA acts as a potent inhibitor of PPP1C and PPP2C catalytic subunits, with considerable selectivity for PPP2C [117,146], greatly advanced our understanding of the roles played by OA sensitive PPPases. OA rapidly become a workhorse inhibitor for the study of PPP family protein phosphatases and their roles in cellular processes. Although, widely reported as a specific or highly selective inhibitor of PP2A, IC<sub>50</sub>s vs PPP2AC, PPP4C, and PPP6C catalytic subunits are all sub-nanomolar (~0.1 nM). PPP1C is less sensitive (15-50 nM IC<sub>50</sub>) and PPP5C has an intermediate sensitivity (~1 nM IC<sub>50</sub>) [138-140,147]. PP2B and PPP7 are far less sensitive than even PPP1C, having IC<sub>50</sub>s above 1 μM [25,146].

The crystal structures of OA in complex with PPP1C $\gamma$  and a PPP2 heterodimer have been reported [94,143]. The PPP1C $\gamma$ :OA structure is shown in Figure 11. From a comparison with the MCLR complex shown in Figure 10 it is readily apparent that the binding sites for the two inhibitors coincide quite closely. Indeed, rather than binding in an extended linear conformation, OA adopts a pseudo-macrocyclic shape, containing two of the inhibitor's three spiroketal moieties, that is stabilized by an intra-molecular hydrogen bond between its C1 carboxylic acid moiety and the C24 hydroxyl. The remaining spiroketal group and a short linking segment form a hydrophobic tail projecting out from the pseudo-macrocycle. OA makes contacts with three absolutely conserved active site residues that are involved in substrate phosphoryl group binding: Arg96, His125, and Arg221. Like MCLR, OA also interacts with residues in and near the hydrophobic groove, principally Ile130, Tyr134, and Trp206. Finally, several residues in the  $\beta$ 12- $\beta$ 13 loop make close contacts with the inhibitor, most notably Tyr272, a conserved residue known from mutagenesis studies to be important for OA binding [148].

The PPP2C:OA complex is shown in Figure 11. The bound conformation of OA is nearly identical to that seen in the PPP1C $\gamma$ :OA complex. Many interactions are shared with the PPP1C $\gamma$ :OA complex. Conserved active site residues Arg89, His118, and Arg214 (Arg96, His125, and Arg221 in PPP1C $\gamma$ ) make similar contacts with OA in both core complexes. Similarly, Tyr265 and Cys266 in the  $\beta$ 12- $\beta$ 13 loop and Ile123, Tyr127, and Trp200 in the hydrophobic groove are seen to recapitulate the interactions of their counterparts in PPP1C $\gamma$ . However, there are non-conserved sequences in the  $\beta$ 12- $\beta$ 13 loop and hydrophobic groove that give rise to differential sets of interactions with the OA pseudomacrocycle and the hydrophobic tail, respectively. In the  $\beta$ 12- $\beta$ 13 loop, PPP1C $\gamma$  has glutamate and phenylalanine side chains contacting hydrophobic groups in a spiroketal moiety within the pseudomacrocycle while in PPP2CA the interacting residues are arginine and cysteine. The polypeptide sequence immediately C-terminal to the conserved (F/W)SA(P/S)NY motif in the  $\beta$ 12- $\beta$ 13 loop abuts the active site and exhibits significant divergence across the PPP family. Thus, divergent sequences in the  $\beta$ 12- $\beta$ 13 loop form very different interacting surfaces for this portion of the inhibitor. Similarly, in the hydrophobic groove, non-conserved residues produce significantly different binding pockets for the OA hydrophobic tail. Gln122 and His191, along with Ile123 and Trp200 (conserved in PPP1C) help form a

hydrophobic cage in the PPP2C:OA complex (but absent from PPP1C) that has much better shape complementarity to the inhibitor (compare in Figure 11).

**1.4.6. Tautomycin:** Tautomycin (TM) is a cytotoxic natural product polyketide produced by the soil bacterium *Streptomyces verticillatus*, having partial structural similarity to okadaic acid [149]. It is a sub-nanomolar inhibitor of PPP1C, PPP2CA/PPP2CB, and PPP4C, a somewhat less potent inhibitor of PPP5C ( $IC_{50} \sim 10$  nM), and a very weak ( $IC_{50} > 10$   $\mu$ M) inhibitor of PP2B/PPP3C [139,149,150].

The crystal structure of the PPP1C $\alpha$ :TM complex was reported by Kelker *et al* in 2008 [151]. TM is found to bind in its diacid form and is shown in Figure 12. Tautomycin can be conceptually divided into two segments: a hydrophobic fragment containing a spiroketal group, which binds in the hydrophobic groove of PPP1C $\alpha$ , and a hydrophilic fragment containing the diacid, as well as other polar groups, which interacts with PPP1C $\alpha$  in and around the active site. The hydrophilic segment forms six hydrogen bonds with four residues in or near the catalytic site: Arg96, Arg221, Val250, and Tyr272. The hydrogen bond with Tyr272 is a feature shared with OA:PPPase complexes and mutation of this residue increases the  $IC_{50}$  of TM by 500-1500-fold [148]. The hydrophobic segment of TM is anchored in the hydrophobic groove through interactions with Cys127, Ser129, Ile133, Tyr134, Trp206, Gly222, and Val223. The reported selectivity of TM for PPP1C vs PPP2CA is quite modest and structural comparisons do not reveal any obvious reasons for selectivity. On the other hand, the markedly lower affinity of TM for PP2B may be due, in part, to substitution of bulky, hydrophobic leucine for a cysteine residue (Cys273 in PPP1C: conserved in all PPPases except PP2B and PPP7) in the  $\beta$ 12- $\beta$ 13 loop. This substitution likely produces a steric clash with the diacid moiety of TM.

**1.4.7. Calyculin A:** Calyculin A (CalA), first isolated from the sponge *Discodermia calyx*, is a cytotoxic agent consisting of a dipeptide segment plus a phosphorylated polyketide moiety [120,121]. It is a potent, sub-nM to low nM, inhibitor of PP1, PPP2C, PPP4C, PPP5C, and PPP6C [138-140,150], with PP2B and PPP7C having much lower affinity ( $IC_{50} > 1$   $\mu$ M). Structure-activity relationship (SAR) studies of CalA derivatives have demonstrated the importance of the phosphate, the C13 hydroxyl, and the polyketide tail, for phosphatase inhibition. The dipeptide segment, however, seems to make only a minor contribution [152].

The PPP1C $\gamma$ :CalA complex structure (Figure 13) was solved by Kita *et al* in 2002 [153]. CalA is observed to make contacts with groups in the active site, the hydrophobic groove, and the acidic groove. The CalA phosphate participates in many polar contacts with the enzyme, interacting indirectly with the active site metals and His125 via hydrogen bonds to structural waters and directly with Arg96, Arg222, and Tyr272 side chains via hydrogen bonds. The hydrophobic tail of CalA (N1-C11) makes numerous non-polar contacts with residues in the hydrophobic groove, including the side chains of Tyr134, Trp206, Ile130, Val223, Cys202, and Asp197. Ser129 and the backbone carbonyl of Arg221 form hydrogen bonds with the C1 nitrile and C11 hydroxyl, respectively. The oxazole moiety makes weak hydrophobic contacts in the acidic groove with Asn219, Asp220, Thr226, Gln249, Asp208, and Asp210. Polar interactions with the enzyme were not observed in the acidic groove. Part

of CalA (C33 to C37) was not modeled due to poor/uninterpretable density and is presumed to contribute little to binding affinity.

**1.4.8. Fostriecin:** Fostriecin is a phosphate monoester isolated from the fermentation beer of *Streptomyces pulveraceus*. The antitumor activity of fostriecin has been evaluated extensively [124,154-156] and marked cytotoxicity against many cancer cell lines as well as potent antitumor activity in animals has been demonstrated. Its potential use as an antitumor agent in humans has been evaluated in Phase 1 clinical trials. However, trials were discontinued before the maximal tolerated dose was established due to the loss of momentum for further development following corporate mergers of the pharmaceutical companies developing fostriecin, which lead to production limitations. There were also concerns raised about storage stability [154]. Fostriecin acts as a potent (~1-3 nM IC<sub>50</sub>) inhibitor of PP2A/PPP2CA [157-159] and PPP4C [138], a weak inhibitor of PPP1C and PPP5C (~50 μM IC<sub>50</sub>) [158, 159] and at 100 μM has no apparent effect on PP2B/PPP3C [138,157-159]. Assessments of fostriecin inhibition of PPP6C and PPP7C have not been reported. Due to its high degree of homology with PPP2CA and PPP4C, PPP6C is expected to be highly sensitive fostriecin. Thus fostriecin appears to be a specific inhibitor of the PPP2/PPP4/PPP6 subfamily, with > 10,000 fold selectivity for PPP2/PPP4/PPP6 vs other PPPases. No structures of fostriecin in complex with PPPases have been reported. However, mass spectrometry analysis of fostriecin:PPP2CA covalent complexes have established that the electrophilic α,β-unsaturated δ-lactone moiety of fostriecin undergoes nucleophilic attack by Cys269 (conserved in PPP2, PPP4, and PPP6) in the β12-β13 loop to form a covalent adduct[160]. Mutagenesis studies and SAR of fostriecin derivatives indicate that the reaction between Cys269 and the lactone is one of the principal determinants of fostriecin's potency and selectivity [158,161]. Mutation of PPP1C or PPP5C to introduce an appropriately placed cysteine dramatically increases fostriecin affinity. In addition derivatives of fostriecin that contain a hydrolyzed lactone experience a 10<sup>5</sup>-fold loss in activity [159]. This provides a high degree of confidence in the positioning of the lactone ring in the active site. However, the position of the hydrophobic tail is not clear at this time.

**1.4.9. Cantharidin and the 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid scaffold:** Cantharidin (2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) is the active constituent in a traditional Chinese medicine. It is naturally occurring in the bodies of ~1500 species of blister beetles, which use it as a chemical defense mechanism [162-164]. Medical use of cantharidin also has a history in Europe where it was first isolated in the early 1800's [165]. Although occasionally utilized as a topical vesicant for the removal of warts [166], cantharidin was considered as obsolete and too toxic for internal use by the early 1900's. Cantharidin acts as a semi-selective inhibitor of PPPases and is known to strongly inhibit the catalytic activity of PPP1C, PPP2CA, PPP5C and PPP6C (IC<sub>50</sub> ~ 0.2–8 μM), while having much lower affinity for PP2BC/PPP3C, PPP4C, or PPP7/PPPEF (IC<sub>50</sub> >> 20 μM) [25,87,133,139,167-169]. Unlike the previously discussed inhibitors, which are all complex natural products that are difficult to synthesize/derivatize, cantharidin class inhibitors, containing a core 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid scaffold, are synthetically accessible and production of derivatives is relatively straightforward.

Structures of cantharidin and related derivatives (e.g. the aquatic herbicide, endothall) in complex with PPP5C have been reported by Bertini *et al* [88]. Some members of this class are synthesized or isolated as anhydrides, while others are diacids. However, only the diacid forms are observed bound in co-crystal structures. Forms with anhydride rings, when incubated in aqueous buffer with enzyme, appear to be readily hydrolyzed to the ring opened diacid. The observed binding modes for inhibitors in this class are all highly similar. As an example, in Figure 14 we show the complex of PPP5C with endothall (PDB 3H61). Endothall is small, with a low molecular weight (186 g/mol) and high ligand efficiency. It binds tightly to PPP5C right at the catalytic center, directly coordinating the metal ions with both carboxylic acids and the bridging O7. One metal ion is pentacoordinate with trigonal bipyramidal geometry and the other maintains an octahedral coordination sphere. The 2- and 3-carboxy groups of endothall also form hydrogen bonds with conserved PPP5C catalytic residues Arg275, Arg400, and His304. A few somewhat unusual interactions are also on display. An anti-parallel dipole-dipole stacking interaction occurs between the 2-carboxyl of endothall and the backbone carbonyl of His427. Three weak CH---O hydrogen bonds help stabilize the position of the oxabicyclo ring: one between the Tyr451 hydroxyl and the C1 hydrogen, one between the C4 hydrogen and the backbone carbonyl of His427, and one between an aromatic ring hydrogen of Phe446 and the bridging oxygen (O7). Also observed are hydrophobic contacts between the inhibitor and side chains of Val429, Phe446, and Tyr451. Almost all of the inhibitor contacts described above are with residues absolutely conserved across the PPP family. The exceptions are Val429 and Phe446, which might account for some of the observed IC<sub>50</sub> differences. Indeed, substituting tryptophan for Phe446 in PPP5C greatly increases the IC<sub>50</sub> for cantharidin and PPP4C, which has a tryptophan at this site, is relatively insensitive [87]. Molecular modeling of endothall bound to PPP4 suggests that the much larger tryptophan side chain may produce a steric clash with the inhibitor that perturbs the high-affinity binding mode it is free to adopt in the PPP5C complex.

### 1.5 Future development of selective inhibitors:

Based on the interactions discussed above, sequence divergence in the  $\beta$ 12- $\beta$ 13 loop, hydrophobic groove, and acidic groove appear to provide opportunities for developing non-conserved contacts in derivatives of existing inhibitors or novel scaffolds. The small size and simple methods needed for the synthesis of nor-cantharidin derivatives makes it an attractive scaffold. However, merely “decorating” the cantharidin core scaffold with small groups may not be adequate. A fragment linking strategy may prove more fruitful. Methods to improve the storage stability of fostriecin may be all that is needed to renew interest in this compound with previously demonstrated marked antitumor activity. Because we now have structural information about how complex natural product inhibitors interact with targeted PPPases, fragments of existing large natural inhibitors that are toxic in their entirety may also provide novel leads for the development of highly specific inhibitors. This may facilitate deriving synthetically accessible fragments from okadaic acid, tautomycin, calyculin A, and microcystin (maybe directly, maybe indirectly via similarity searches on interesting substructures) that could be linked up to a core scaffold.



It may also prove useful to conduct virtual screening and biophysical screening (SPR, NMR, MS etc.) to identify compounds with improved selectivity. As the structures of PPP1 and PPP2 holoenzymes are reported, it will likely be fruitful to explore the development of compounds that interact at the interface where the substrate, catalytic and regulator subunits converge. Such compounds will likely demonstrate greatly reduced systemic toxicity and thus have better target specific utility.

## CONCLUSION:

Although PPP-family phosphatases share a common catalytic mechanism, insights obtained from the examination of PPPase-inhibitor co-crystal complexes indicate that the development of more selective or even specific inhibitors should be possible.

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## Biography



## LIST OF ABBREVIATIONS

<b>PPPase</b>	PPP-family serine/threonine protein phosphatase
<b>OA</b>	okadaic acid
<b>MCLR</b>	microcystin-LR
<b>CalA</b>	calyculin A
<b>TM</b>	tautomycin

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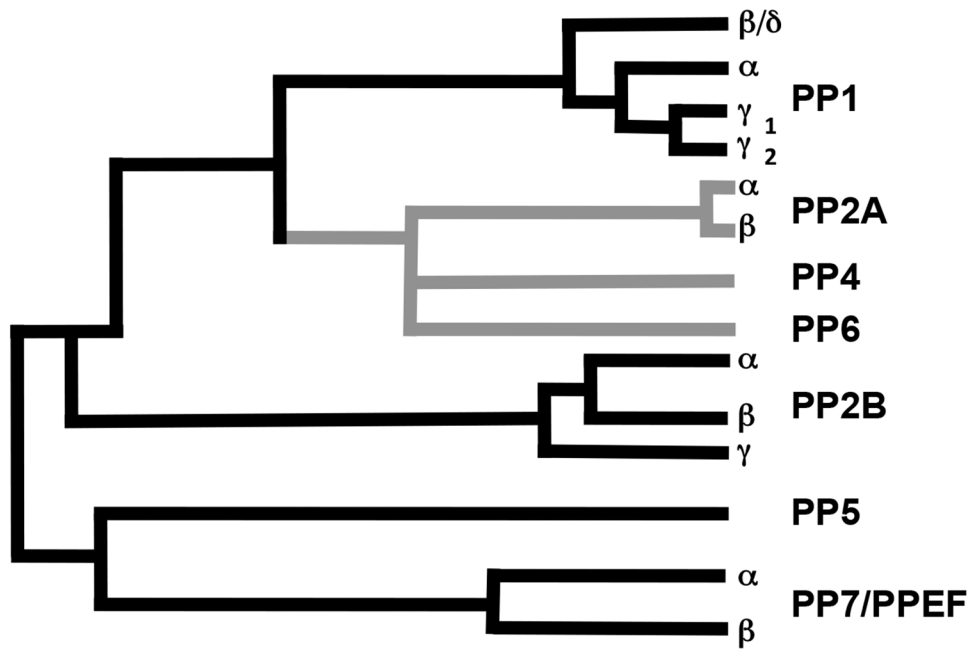


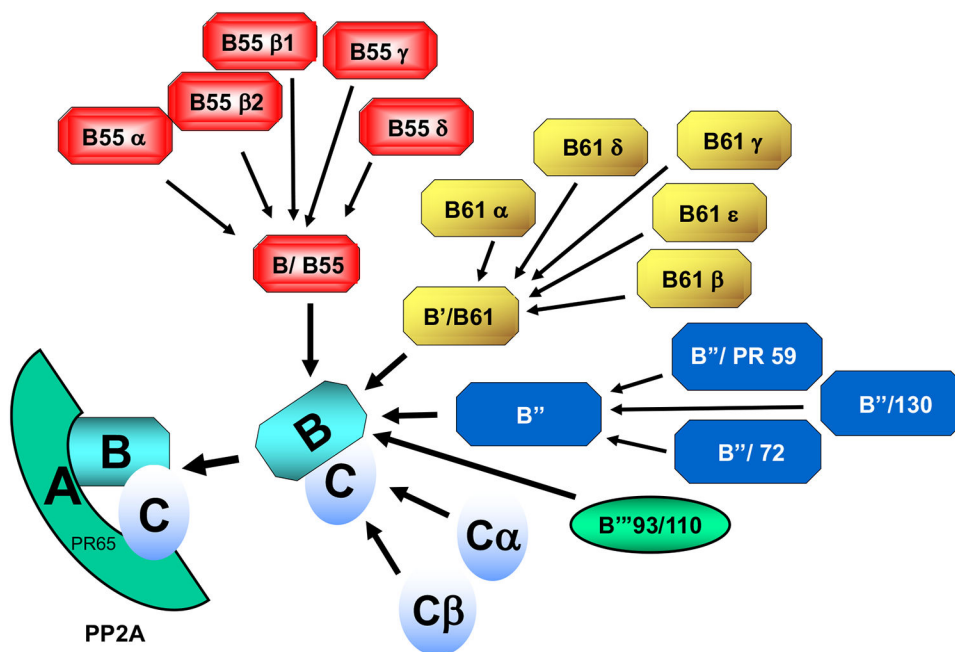
Figure 1. Mammalian branch of the PPP-family

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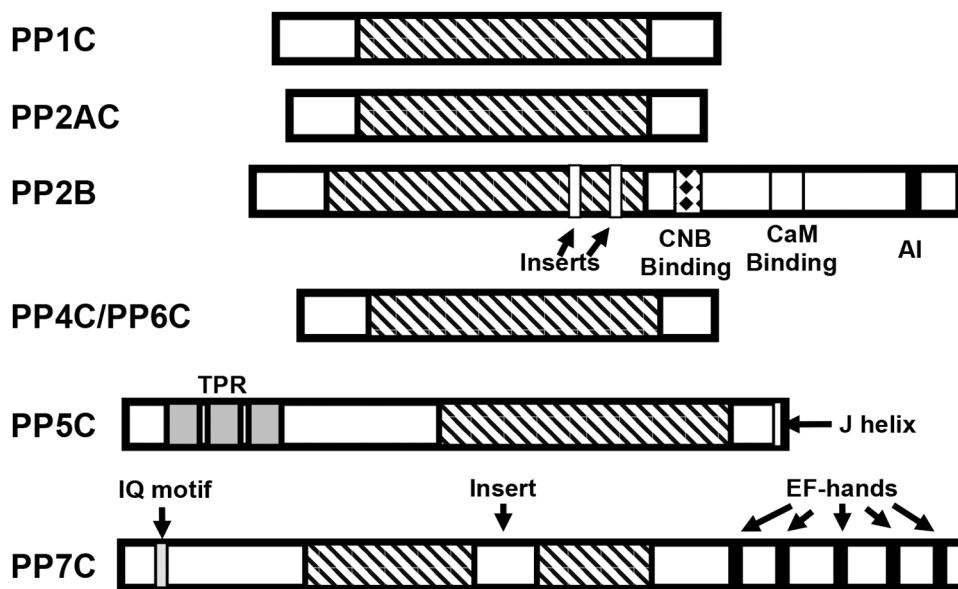
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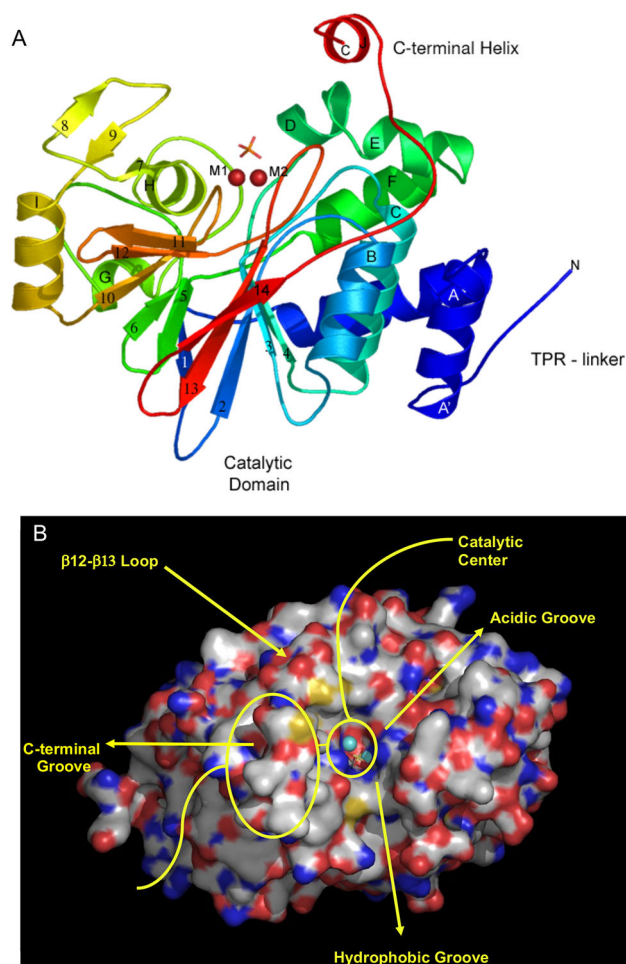


**Figure 2.** Myriad of PP2A/PPP2-holoenzymes generated from different combinations of catalytic and regulatory subunits. PP2A-holoenzymes are composed of a catalytic subunit (C) a scaffold protein (A/PR65) and any one of several targeting/regulatory proteins (B). There are two isoforms of the A and two isoforms of the C ( $\alpha$ ,  $\beta$ ) subunits. The B-regulatory/targeting subunits are derived from one of four structurally unrelated families (B/B55; B'/B61; B''; B'''/93/110). Each of the different B families contains several isoforms, some of which are expressed in a tissue or developmentally specific manner. Given the existence of two isoforms of A, two isoforms of C and at least 18 different B subunits, >75 different trimeric forms of PPP2 can be generated with the known proteins. Table 1 provides a cross reference for the old and new (assigned based on the gene) names for the A, B and C subunits

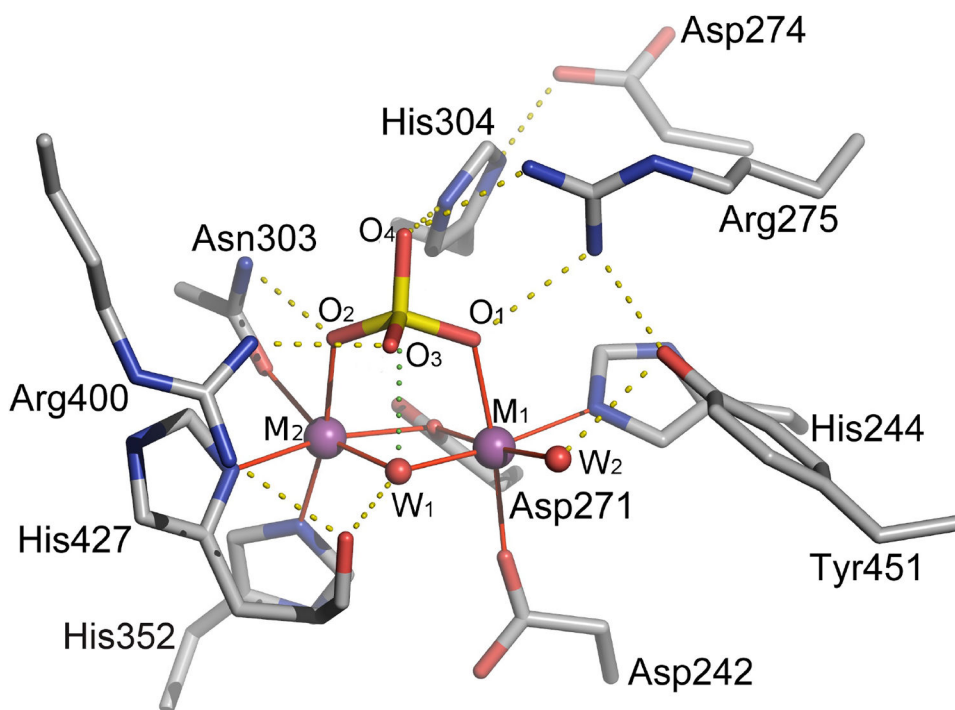


**Figure 3. Homology and domain organization of PPP-family phosphatases.**

PPPase share a highly conserved catalytic core domain of ~250 amino acids (indicated by diagonal stripes). PPP1, PPP2, PPP4, and PPP6 catalytic subunits are highly homologous enzymes, differing primarily in their C- and N-terminal regions. PP2B catalytic subunits (calcineurin A) differs in that it has a large C-terminal extension containing a regulatory subunit (calcineurin B; CNB) binding site (indicated by a diamond filled box), a Ca<sup>2+</sup>-calmodulin (CaM) binding domain (indicated by a white box), a short autoinhibitory (AI) domain in its C-terminal region and two small divergent regions (inserts) in the catalytic core. PPP5C possesses three tetratricopeptide repeats (TPRs - indicated by grey boxes) in its N-terminal region and a short regulatory helix (J helix; indicated by a white box) at its C-terminus. PPP7 contains a CaM binding IQ motif (gray box) in its N-terminal region, Ca<sup>2+</sup> binding EF-hand motifs (indicated by black boxes) in its C-terminal region and a 43-amino acid insert in the catalytic core domain (indicated by an open box).

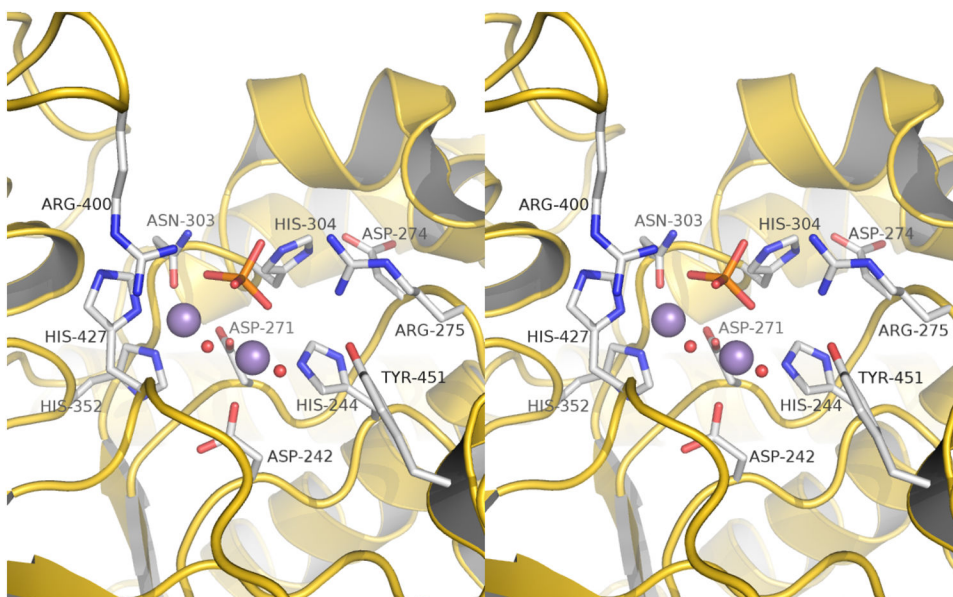


**Figure 4.** Structure of the PPP-phosphatase fold as represented by PPP5C. The catalytic domain of serine/threonine protein phosphatase in complex with orthophosphate (PDB code: 1S95) provides an example of the overall structure of the phosphatase domain. **A)** A cartoon representation of the PPP5C structure with is shown in rainbow colors starting with blue at the N-terminus and ending with red at the C-terminus. Divalent metal ion cofactors,  $M_1$  and  $M_2$  (red spheres) and a phosphate ion indicate the active site location. Alpha helices are labeled A' to J and beta strands are labeled 1 to 14. PPP5C specific features, such as the C-terminal J helix and the TPR domain linker, are also labeled. **B)** Surface representation of the structure of PPP5C derived from PDB code 1S95. Bound orthophosphate is displayed as sticks, and the catalytic metal ions are depicted as spheres.



**Figure 5. A detailed representation of contacts within the active site.**

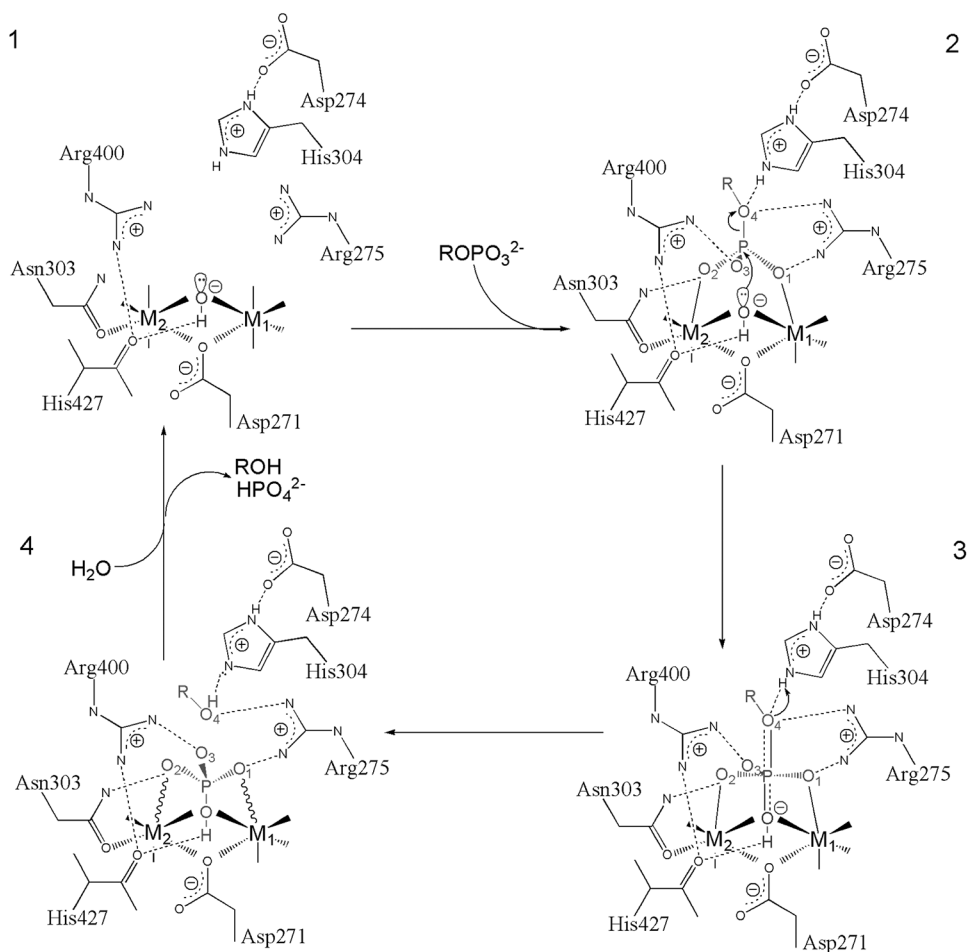
Hydrogen bonds are shown as dotted yellow lines and coordination bonds to metal ions are shown as solid red lines. The dotted green line between the hydroxide ( $W^1$ ) ion and the phosphorus atom (P) represents a close contact suggestive of the near-attack configuration in metal ion-mediated hydrolysis of phosphoprotein substrates. Adapted from Swingle *et al* 2004 [68].



**Figure 6. Catalytic site of PPP5C.**

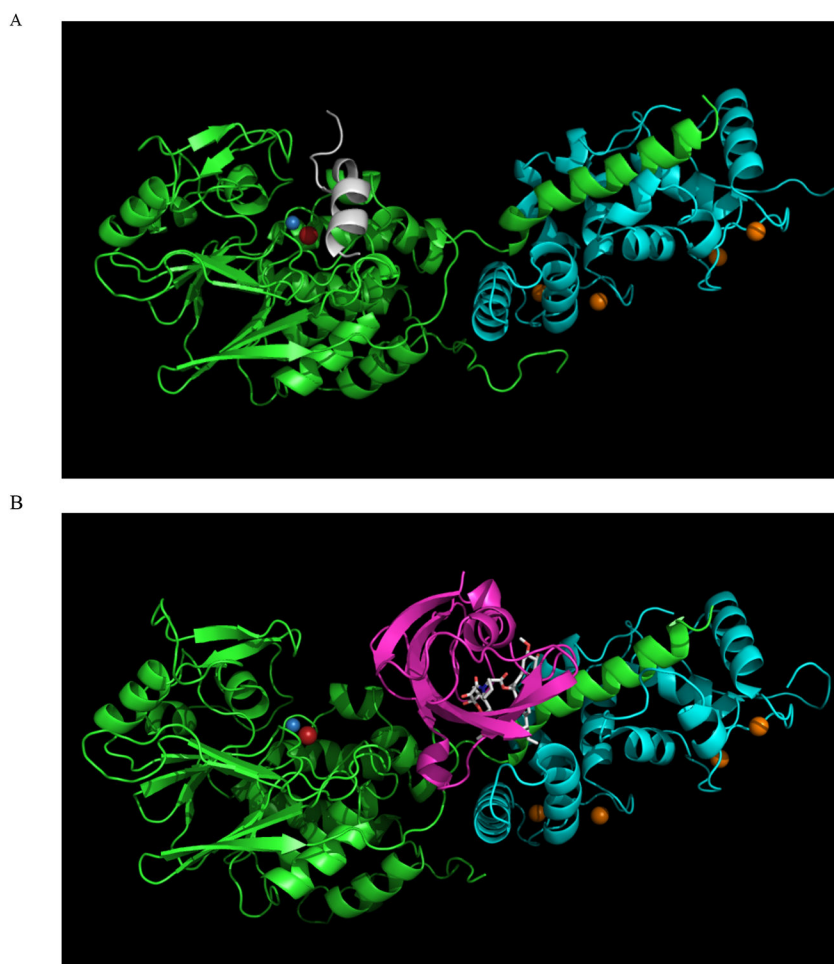
A stereo representation of the active site of the catalytic domain of PPP5C. Pertinent active site residues are labeled and shown in stick representation with carbon atoms colored white, oxygen atoms colored red, nitrogen atoms colored blue and phosphorus atoms colored orange. The active-site metal ions are shown as purple spheres and metal ligated waters are shown as small red spheres. Adapted from Swingle *et al* 2004 [68].



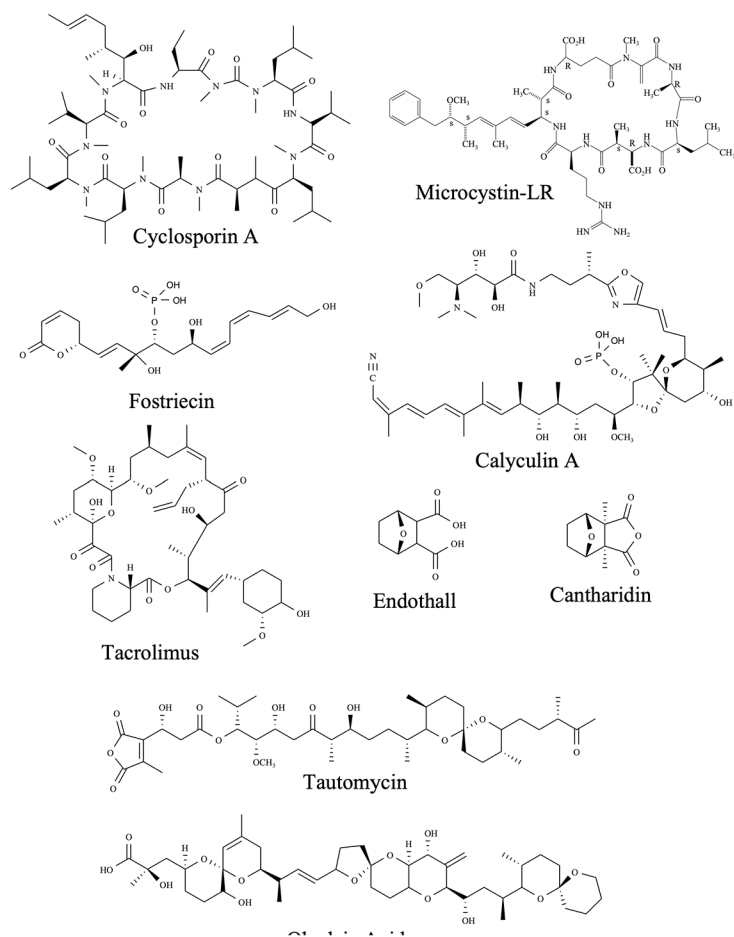


**Figure 7. PPPase Reaction mechanism.**

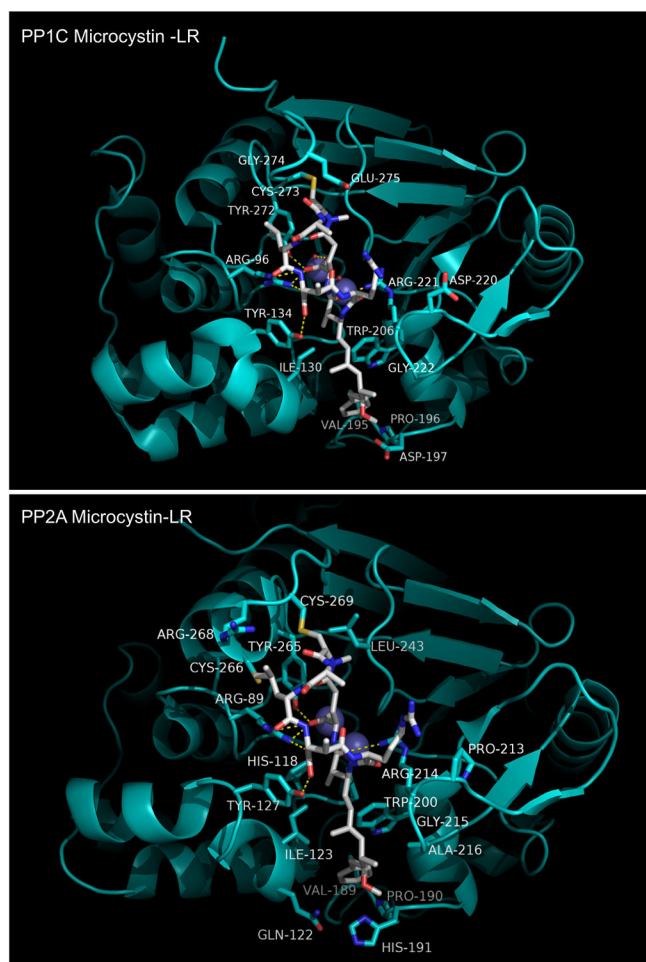
A Schematic representation of phosphomonoester hydrolysis mediated by metal ions and active site residues is presented. PP5 residue numbering is used. Solid lines to the metal ions denote metal-ligand bonds, and solid or dashed wedges indicate metal-ligand bonds directed above or below the plane of the page, respectively. Wavy lines to the metal ions indicate strained contacts with poor coordination geometry. Dotted lines indicate hydrogen bonds, and the nearly dissociated axial bonds in the transition state are shown by half-dotted double-lines. Adapted from Swingle et al. the Journal of Biological Chemistry 2004 [68].



**Figure 8. Inhibition of PP2B by immunophilin/immunosuppressant complex.**  
Inhibition of PP2B by immunophilin/immunosuppressant complex. A. The structure of a PP2B holoenzyme is shown in a cartoon representation. The PP2B catalytic subunit is green, the regulatory B subunit is cyan, and the autoinhibitory domain is white. Metal ions are shown as spheres: Fe is red, Zn is blue, and Ca is orange. B. The structure of a PP2B holoenzyme bound to the inhibitory FKBP12:tacrolimus complex is shown in a cartoon representation. The PP2B catalytic subunit is green, the regulatory B subunit is cyan, and FKBP12 is magenta. Metal ions are shown as spheres: Fe is red, Zn is blue, and Ca is orange. Tacrolimus (FK506) is shown as sticks



**Figure 9. Natural compounds with inhibitory activity against PPP-family phosphatases**



**Figure 10. Microcystin-LR bound to PP1C and PP2A**

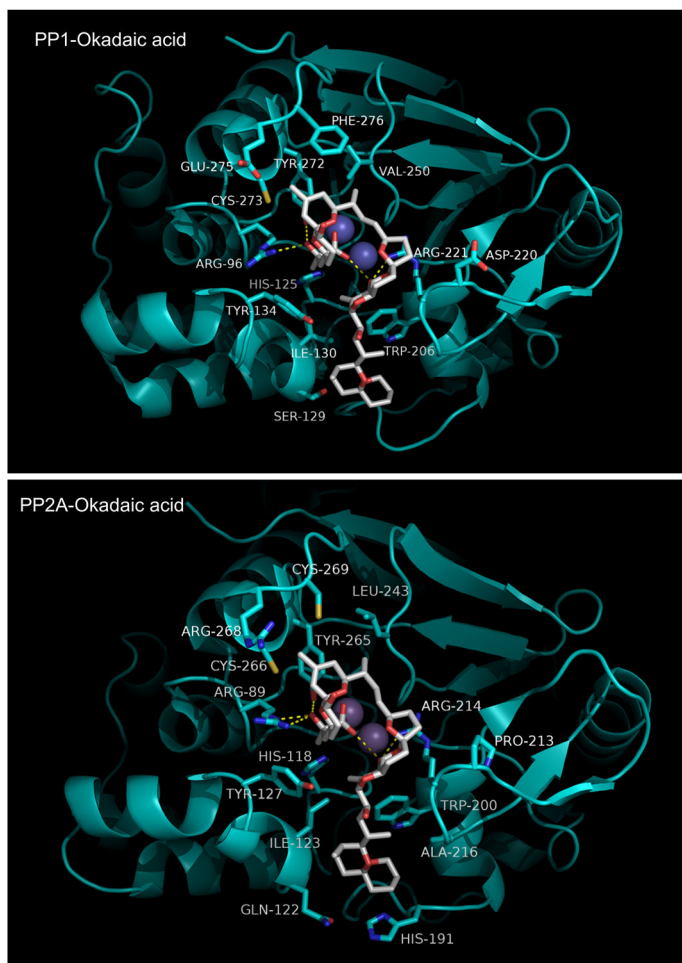
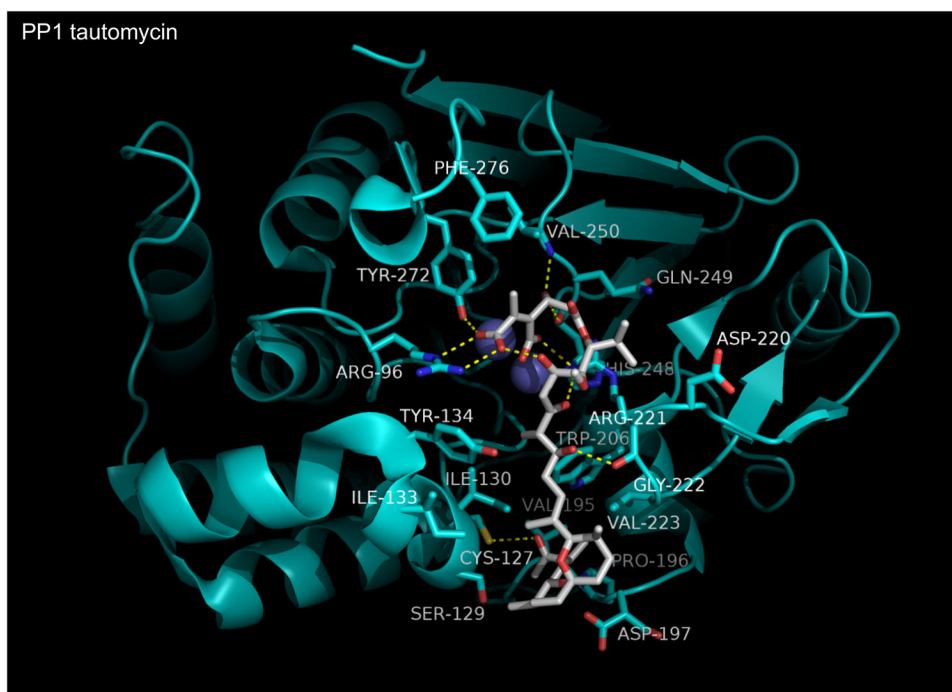
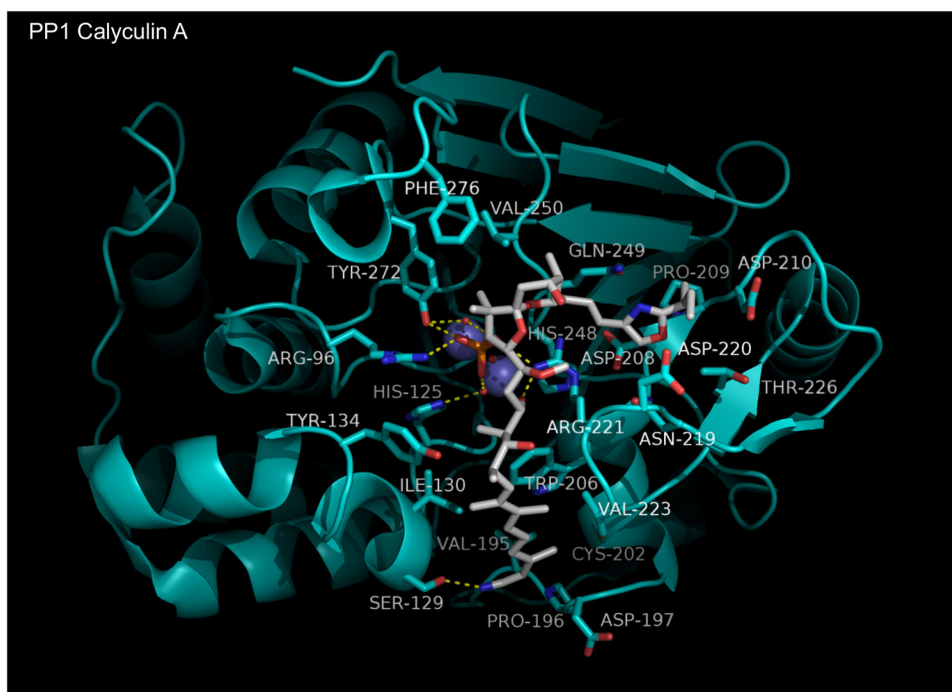


Figure 11. Okadaic acid bound to PP1C and PP2A



**Figure 12.** Tautomycin bound to PPP1C



**Figure 13.** Calyculin A bound to PPP1C

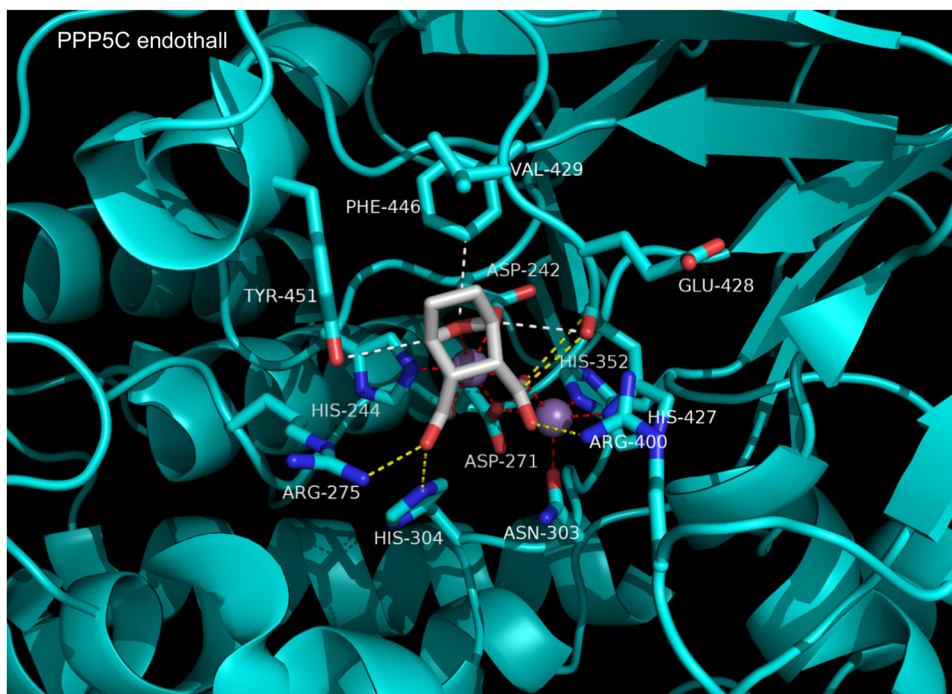


Figure 14. Endothall bound to PPP5C



**Table 1.**

Nomenclature associated with PPP2-phosphatases

Subunit	Gene	Isoform	Other Name(s)
Scaffold (A)	PPP2R1A	$\alpha$	PP2AA $\alpha$
Scaffold (A)	PPP2R1B	$\beta$	PP2AA $\beta$
Catalytic (C)	PPP2CA	$\alpha$	PP2A $\alpha$
Catalytic (C)	PPP2CB	$\beta$	PP2A $\beta$
Regulatory (B)	PPP2R2A	$\alpha$	B55 $\alpha$ , PR55 $\alpha$ , PP2AB $\alpha$
Regulatory (B)	PPP2R2B	$\beta$	B55 $\beta$ , PR54 $\beta$ , PP2AB $\beta$
Regulatory (B)	PPP2R2C	$\gamma$	B55 $\gamma$ , PR55 $\gamma$ , PP2AB $\gamma$
Regulatory (B)	PPP2R2D	$\delta$	B55 $\delta$ , PR55 $\delta$ , PP2AB $\delta$
Regulatory (B')	PPP2R5A	$\alpha$	B56 $\alpha$ , PR56/61 $\alpha$ , PP2AB' $\alpha$
Regulatory (B')	PPP2R5B	$\beta$	B56 $\beta$ , PR56 $\beta$ /PP2AB' $\beta$
Regulatory (B')	PPP2R5C	$\gamma, 1, 2, 3$	B56 $\gamma$ , PR56/61 $\gamma$ , PP2AB' $\gamma$
Regulatory (B')	PPP2R5D	$\delta$	B56 $\delta$ , PR56/61 $\delta$ , PP2AB' $\delta$
Regulatory (B')	PPP2R5E	$e$	B56 $e$ , PR56/61 $e$ , PP2AB' $e$
Regulatory (B'')	PPP2R3A	$\alpha$	PR130, B'' $\alpha$ 1
Regulatory (B'')	PPP2R3A	$\alpha$	PR72, B'' $\alpha$ 2
Regulatory (B'')	PPP2R3B	$\beta$	PR70, PR48, B'' $\beta$
Regulatory (B'')	PPP2R3C	$\gamma$	G5PR, G4-1
Regulatory (B'')	PPP2R3D	$\delta$	PR59, B'' $\delta$
Regulatory (B''')	PPP2R6A	STRN	Striatin, PR110,
Regulatory (B''')	PPP2R6B		STRN3, SG2NA
Regulatory (B''')	PPP2R6C		STRN4
Regulatory (B''')	PPP2R4		PTPA, PR53