Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 9, pp 1739–1750, 1997

PII: S0968-0896(97)00146-6
0968-0896/97 $17.00 + 0.00

Review Article

Inhibition of the Ser–Thr Phosphatases PP1 and PP2A by Naturally Occurring Toxins

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1. Introduction: The Protein Phosphatases

The reversible phosphorylation of proteins containing serine, threonine, and tyrosine amino acids is a widely recognized mechanism that regulates many cellular processes (Fig. 1). Phosphorylation of these hydroxyl-bearing amino acid side-chains is catalyzed by the protein kinases (PKs) using ATP as a phosphoryl donor, whereas dephosphorylation is catalyzed by the protein phosphatases (PPs).

This simple molecular cycle acts as an 'on–off' switch to selectively modulate the action of countless other proteins and is ubiquitous in eukaryotic cells. Protein (de)phosphorylation induces changes in protein conformation, protein–protein interactions, protein–ligand interactions, membrane permeability, and solute gradients, among others. The culmination of these physicochemical changes is the regulation of cellular processes such as glycogen synthesis, cell division, gene expression, neurotransmission, muscle contraction, and a plethora of other second messenger and signal transduction pathways, the importance of which cannot be understated.[1,2]

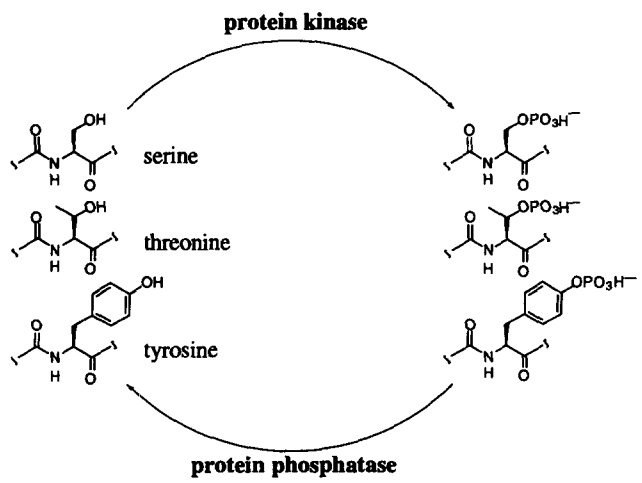


Figure 1. Phosphatase/kinase cycle.

The number of phosphatases discovered to date is presently over 100 but estimates place the total number at ~1000 or >1% of the coded human genome![3] The sheer quantity of PPs and the mechanisms of phosphate hydrolysis that they exhibit are as numerous and rich as the proteases. Not surprisingly, the number of protein kinases complements the hundreds of phosphatases, the sum of which provides finely tuned and complexly regulated homeostasis in cells.

Because the kinases and phosphatases affect other proteins and literally have hundreds of substrates, it has been a formidable challenge to decipher these complex pathways, which are only now beginning to be understood. Recent X-ray crystal structures for protein kinase A (PKA or cAMP-dependent protein kinase)[4,5] and protein tyrosine phosphatase 1B (PTP1B)[6] have contributed greatly to a detailed understanding of the function and regulation of these biological catalysts. For the kinases, a useful paradigm for deducing specifically which enzymes target which substrates comes from the consensus sequence of the latter. For instance, the consensus sequence for PKA is a substrate which possesses an RRXS(T)Y sequence where X is a small residue and Y is a large hydrophobic residue.[7,8]

Prior to 1988, there were four major serine/threonine protein phosphatases known to be present in eukaryotic

cells: PP1, PP2A, PP2B (calcineurin, Ca^{2+}/calmodulin-dependent protein phosphatase), and PP2C (ATP/Mg^{2+}-dependent protein phosphatase). More recently, this list has been expanded to include newly discovered relatives of PP1 (PPZ1, PPZ2, PPQ), PP2A (PP4, PPV, PPG), and PP2B (rdgC) as well as a new subfamily dubbed PP5.[1,9,10] In addition, new isoforms of the above enzymes are continually being discovered.

The holoenzyme structures of PP1 and PP2A in vivo are complex as is their regulation and substrate specificity. Both phosphatases contain a catalytic subunit ($PP1_C$ and $PP2A_C$, 37 and 36 kDa, respectively) that is structurally related and shares 50% amino acid identity. Structural homology of these enzymes is remarkably conserved even between phyla. For instance, mammalian and *Drosophila* PP1 show ~90% sequence identity while mammalian and yeast exhibit >80% identity—one of the most homologous classes of enzymes known.[11] The catalytic subunit of both enzymes can further associate with either one or two additional regulatory subunits (called targeting subunits) that affect $PP1_C/2A_C$ by attenuating their substrate specificity, modulating their activity, and localizing them to specific subcellular locations.[12] PP2A is typically a heterotrimer of A, B, and C subunits, in vivo, and several forms of the A and C subunits as well as many variants of the B subunit, have been identified. Although the B subunit is thought to be largely responsible for distinct substrate specificity, the regulatory roles of the targeting subunits remains poorly understood. PP1 is found associated with myosin, glycogen, the nuclear chromatin, and the cell membrane of the sarcoplasmic reticulum where it controls a variety of cellular functions. PP2A was thought to reside primarily in the cytoplasm, but is now found distributed in many tissues.

In addition to regulation by the targeting subunits, PP1 and PP2A are also stringently regulated by six endogenous protein inhibitors. Inhibitor-1 (I-1), Inhibitor-2 (I-2), dopamine and cAMP-regulated phosphoprotein (DARPP-32),[1] and nuclear inhibitor of protein phosphatase 1 (NIPP-1)[13-16] specifically inhibit PP1 but do not affect the other phosphatases. Historically, PP1 was differentiated from PP2A based on sensitivity to I-1 and DARPP-32. Very recently, two proteins, $I\text{-}1^{PP2A}$ [or PHAP-I (putative histocompatability leukocyte antigens class II associated protein I)] and $I\text{-}2^{PP2A}$ (also SET, or PHAP-II) have been determined to specifically inhibit PP2A without affecting the other phosphatases.[12,17,18] Additionally, more regulation of PP1 and PP2A occurs indirectly via activation and deactivation by secondary messengers such as cAMP, ATP, and glucose, but with different specificities. Lastly, PP2A has been shown to be deactivated by phosphorylation on threonines and tyrosines and activated by carboxy-terminus methylation.[19-21] The atomic structure of PP1 was recently revealed by X-ray crystallography independently by two groups.[22,23]

PP2B, or calcineurin, is a heterodimer comprised of two subunits that share some overlapping substrate specificity with PP1 and PP2A. Calcineurin A, a 60 kDa catalytic subunit, is highly homologous (~40% identical) to the catalytic subunits of PP1/2A but is larger since it contains an additional 170 *C*-terminal residues comprising a calmodulin-binding domain. Calcineurin B, is a 19 kDa regulatory subunit that bears four putative Ca^{2+}-binding sites, similar to those found in calmodulin (CaM) and is regulated by both Ca^{2+} and CaM. Calcineurin is also thought to be regulated by the FK506-binding proteins and cyclophilins (although the endogenous ligands thought to be mimicked by FK506 and cyclosporin have not yet been identified). In recent years, calcineurin has received considerable attention due to its role in T cell activation and immunosuppression and an X-ray crystal structure of a calcineurin/FKBP12/FK506/PO_4^{2-} complex was solved independently by researchers at Vertex Pharmaceuticals[24] and Agouron Pharmaceuticals.[25] PP2C is a monomeric protein that is non-homologous to any of the phosphatases found thus far, but it does exhibit some overlapping substrate specificity with the others. It is localized exclusively in the cytoplasm and is implicated as a regulator of cholesterol synthesis but has not been studied extensively.[2] PP4 (PPX) was discovered in the centrosomes of cells by Cohen and co-workers[26] and is 65% identical to PP2A and 45% identical to PP1 in its catalytic domains. PP5 is <45% related to the PP1/2A/2B family and is located in all examined human tissues, predominantly in the nucleus.[9]

In contrast to many enzymes, including the kinases above, the Ser–Thr-specific PP family exhibits broad and overlapping substrate specificity (especially PP1 and PP2A) with no apparent substrate consensus sequence. Thus, discerning which phosphatase is responsible for controlling a particular cellular pathway has by no means been a trivial task, and biologists have often relied on naturally occurring small molecule toxins to achieve this goal.

2. Learning by Inhibition

Without question, much of the present knowledge of the Ser–Thr-specific PPs has grown out of studies involving their inhibition. Many naturally occurring compounds of strikingly diverse structure have been identified that either selectively or specifically inhibit the phosphatases.[27] A prominent example is the work of Schreiber and others, who have elegantly clarified the inhibition of calcineurin (PP2B) by immunophilin/immunosuppressive natural product dimers.[28,29] The complex of FK506 and the FK506 binding proteins as well as cyclosporin–cyclophilin complex was shown to potently and specifically inhibit calcineurin leading to immunosuppression. Further study revealed that calcineurin is responsible for dephosphorylating the nuclear factor of activated transcription ($NF\text{–}AT_p$) which, together with other transcription factors, promotes the transcription of the interleukin-2 gene—a critical step

in the proliferation of T cells. Cyclosporin and FK506, as well as calmodulin antagonists, have become invaluable tools in molecular biology as specific calcineurin inhibitors for establishing the role of calcineurin in cellular regulation. Similar to calcineurin, the action of PP2C has been specifically targeted using nonhydrolyzable ATP mimics and antagonists.[30] Thus, researchers are compiling an arsenal of small molecules that will enable the inhibition of specific members of the phosphatase manifolds.

Although it is now easy to distinguish the action of PP2C and calcineurin from PP1 and PP2A, it has been much more difficult to distinguish between PP1 and PP2A. The history behind PP1/2A inhibition is even richer than the calcineurin story.

2.1. The endogenous protein inhibitors

As mentioned above, six endogenous proteins have evolved to stringently regulate the action of PP1 and PP2A by specifically inhibiting them without affecting the other Ser–Thr phosphatases (Fig. 2).[27]

The discovery of Inhibitor-1 by Huang and Glinsmann in 1975 marked a turning point in the study of protein phosphatases.[1] This inhibitory protein (as well as its homolog DARPP-32) differentiated the homologous PP1 and PP2A from one another and has since been used in vitro for that purpose. However, it should be emphasized that their utility as specific PP1 inhibitors is limited because they are proteins and require micro-

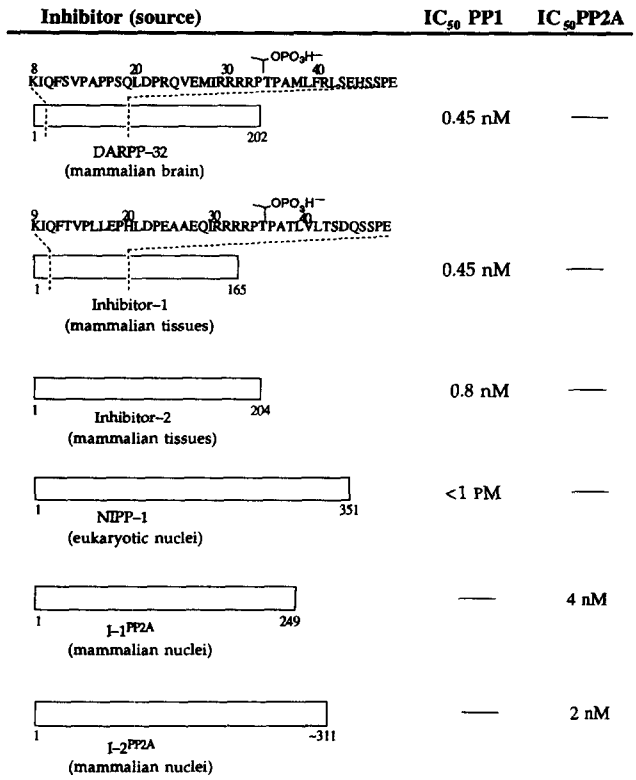


Figure 2. Known endogenous protein inhibitors of PP1 and PP2A.

injection into cells where they are vulnerable to protease catabolism and inactivation by dephosphorylation (for DARPP and I-1) by PP2A and PP2B.

Structural studies by Greengard et al. on I-1 and its isoform DARPP-32 show a highly conserved region from position 9 to 48 (DARPP numbering) and that phosphorylation of threonine 34 by protein kinase A is a prerequisite for potent inhibition. Although these inhibitors consist of 165 and 202 amino acids respectively, it has been shown that the 8–38 fragment retains comparable activity to full-length DARPP (IC_{50} of 40 and 0.5 nM, respectively).[31,32] The remote Ile9 was also shown to be crucial for inhibition perhaps because it may contribute to secondary structure in the vicinity of the phosphorylation site since residues 8–12 are predicted to have β-sheet structure.[33] When recombinant DARPP-32 was used, non-phosphorylated DARPP still inhibited PP1 with an IC_{50} of 1 μM making it essentially equipotent with mutants possessing alanine or glutamate in place of the phosphothreonine.[34]

Despite the high homology among PP1, PP2A, and PP2B, phosphorylated I-1 and DARPP are *inhibitors* of PP1 but *substrates* of PP2A and PP2B. The reasons for this difference are not known; however, phosphothreonine-containing peptides with flanking prolines are dephosphorylated by PP2A much more slowly than other substrates (but Mn^{2+} ions counteract this effect).[35] By contrast, increasing the number of arginines *N*-terminal to phosphothreonine increases the dephosphorylation rate by PP2A.[30] By replacing the phospho-Thr in truncated DARPP with phospho-Ser, its inhibition of PP1 is dramatically diminished 25-fold whereas the dephosphorylation rate by PP2A is decreased less than fourfold, and not decreased at all by calcineurin.[32]

Inhibitor-2 (I-2) is an endogenous inhibitor completely unrelated to the others. It is thought to be to be a chaperone for PP1 because recombinant PP1 is Mn^{2+}-dependent unless treated with I-2, glycogen synthase kinase 3 (GSK-3), and ATP whereby it undergoes a conformational change to become indistinguishable from native (Mn^{2+}-independent) PP1 although its function as a chaperone in vivo has been disputed.[10,36,37] Interestingly, treatment of native PP1 with I-2 alone, or after prolonged storage, induced reversion to a Mn^{2+}-dependent form implying that in vivo, $PP1_C$ appears to be a kinetically folded protein.

The nuclear inhibitor of PP1 (NIPP-1) was discovered bound to the chromatin of the nucleus. Unlike DARPP-32 and I-1, NIPP-1 requires *dephosphorylation* to become an active phosphatase inhibitor. Sequencing shows this protein to be homologous to mRNA trafficking proteins and myosin, the latter being a known regulatory ligand for PP1.[13–16]

The proteins $I\text{-}1^{PP2A}$ [also termed PHAP-I (putative histocompatability leukocyte antigens class II associated protein I)] and $I\text{-}2^{PP2A}$ [also called SET, PHAP-II, and

TAF (template-activating factor)] were only recently deduced to be specific PP2A inhibitors.[12,17,18] Only the sequence of these proteins has been determined, and it is unknown if they require phosphorylation to become (in)active. These two inhibitors share an extensively acidic *C*-terminus but are unrelated to each other (and the other protein inhibitors) and bind PP2A noncompetitively with one another.

Although the protein inhibitors above are mechanistically informative about how the protein phosphatases might be inhibited, they are handicapped by the inherent shortcomings of peptides in general: proteolytic degradation; poor membrane permeability (e.g., the blood–brain barrier); high molecular weight; potential instability; and often an inability to obtain a practical quantity of protein. Thus, the medicinal chemists' credo of 'peptides don't make drugs' extends to their use as in vivo inhibitors as well.

The solution to this dilemma has been the use of small molecule inhibitors that are not compromised by the problems faced by peptides. Many naturally occurring structural scaffolds such as alkaloids, terpenes, oligosaccharides, and polyketides have evolved to mimic and/or compliment small areas of the three-dimensional protein–peptide molecular surfaces. Using random screening assays of pharmacologically active or interesting compounds from a variety of organisms, researchers have identified many natural products that inhibit the serine/threonine-specific protein phosphatases as described below.

2.2. The okadaic acid class of inhibitors

The cyclic peptides. Blue–green algae produce many variants of two related cyclic peptide toxins, the microcystins and the nodularins.[38–40] The toxic side-effects of these inhibitors have been known for centuries. The consequences of ingesting brackish water where blue–green algae bloom have been documented in historical accounts as one such article published in an 1878 issue of *Nature* attests:[41]

> Symptoms—stupor and unconsciousness, falling and remaining quiet, as if asleep, unless touched, when convulsions come on, with head and neck drawn back by rigid spasm, which subsides before death. Time—sheep, from one to six or eight hours; horses, eight to twenty-four hours; dogs, four to five hours; pigs, three or four hours.

Human and animal poisonings continue to this day and concern toxicologists because of the increasing frequency of algal blooms due to fertilizer runoff. The microcystin-XY family are cyclic heptapeptides which possess several D-amino acids, isolinked glutamate and β-methylaspartate residues, dehydroalanine, and an unusual aromatic amino acid abbreviated Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-deca-dienoic acid]. Many naturally occurring congeners have been isolated in which the amino acids at positions X and Y of the macrocycle bear other L-amino acids. For example, microcystin-LA (Fig. 3) has leucine and alanine in these positions.

The nodularins are analogous cyclic pentapeptides in which the 'X' amino acid and the adjacent alanine have been excised from the macrocycle.[42–44] Like the microcystins, they too possess a variable 'Y' position and it is of interest that nodularin-V (named motuporin in the literature[45]) was actually isolated from a marine sponge—a fascinating testament to either convergent evolution or perhaps the predation of these sponges on marine algae that also synthesize these toxins. These cyclic peptides are 'suicide' inhibitors since they are known to covalently modify the phosphatases via Michael addition of a nucleophilic cysteine in the protein to the dehydroalanine in the inhibitors.

Structure–activity studies have been conducted with natural microcystin variants (Fig. 3) and non-natural molecules produced by simple chemical modification. Among the natural analogues, microcystins-LR and -LA are more toxic to adult mice than are microcystins-LY and -RR (LD_{50} values 36, 39, 91, and 111 ng/g, respectively) although the pathophysiological responses induced by each microcystin are identical.[46] Other known microcystins span a range in potency in adult mice from 50 to 200 ng/g.[47] Saturation of the dehydro residue has been shown to have little effect on compound toxicity while esterification of the acid functionality of the Glu residue is known to attenuate toxicity.[48] Other studies have indicated that Adda, nontoxic in free amino acid form, plays an important role in phosphatase inhibition by microcystins since hydrogenation or ozonolysis of the alkenoic positions attenuates activity as does the presence of a *cis* (rather than *trans*) geometry about the Δ^6 double bond.[40,49] However, one strain of microcystis produces a compound containing a modified Adda unit wherein the methoxy substituent at the C-9 position is replaced by an acetoxy group with no significant decrease in toxicity.[50–52] Likewise, a natural product analogue of microcystin containing a C-9 hydroxyl in place of the methoxy substituent of Adda was nearly as toxic as the

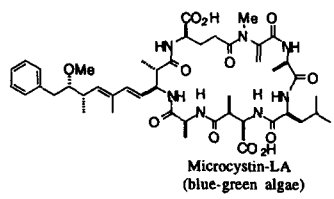
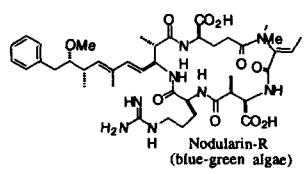
Inhibitor (source)	IC_{50} PP1	IC_{50} PP2A
 Microcystin-LA (blue-green algae)	0.1 nM	0.1-1 nM
 Nodularin-R (blue-green algae)	0.1 nM	0.1 nM

Figure 3. Known cyclic peptide inhibitors of PP1 and PP2A.

parent compound itself.[40,52] As of 1995, more than 40 different microcystins had reportedly been isolated from *Microcystis*, *Anabaena*, *Nostoc*, and *Oscillatoria*.[53] In addition to simple amino acid substitution at the variable X and Y positions and modified Adda residues, several other interesting structural variants are known. These include: Ser[7] microcystin-LR (L-serine in place of Mdha at position 7); Ser[7] microcystin RR; the non-*N*-methyl derivatives Dha[7] microcystin LR and Dha[7] microcystin RR as well as desmethylated versions at D-β-MeAsp.[54] Some of these variants could be biosynthetic precursors to the parent toxins, an idea that is supported by the fact that Ser residues are known biological precursors to dehydroalanine residues.[55] Although many different microcystins have been isolated, the structure–activity information is restricted by the small quantities of toxins available through isolation, and by the limited types of chemical transformations that can be performed on the natural products themselves.

Like the microcystin-XY series, the nodularins (Fig. 3) also have variable regions. To summarize the important features among this group: esterification of the isoglutamate carboxyl destroys activity, change of Adda's MeO to HO decreases toxicity twofold, changing D-MeAsp to D-Asp results in no loss of toxicity, and isomerization of Adda's trisubstituted olefin from *E* to *Z* abolishes activity as does scission of the macrocycle between the L-Arg and Adda.[56]

The terpenoids. Two of the more recently recognized PP inhibitors are both terpenoids (Fig. 4). Thyrsiferyl-23-acetate (TA) was discovered in 1985[57] but the parent skeleton was structurally determined in 1978.[58] TA is biosynthesized directly from squalene, presumably via a bromonium ion-mediated pathway.[59] Although it is far less potent than the other PP inhibitors, it is the most selective small molecule PP2A inhibitor discovered to date.[60] No structural studies have been performed on TA.

Cantharidin, like the cyclic peptides, has a rich history. It is a powerful vesicant used in the 1800s to remove warts, but its high toxicity limited general use.[61] Human poisonings continue to this day as the result of folklore surrounding its purported aphrodisiac ('Spanish fly')

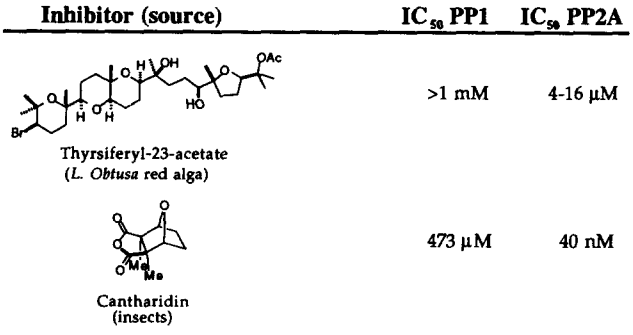


Figure 4. Known terpenoid inhibitors of PP1 and PP2A.

and abortifacient properties.[62] This *meso*-symmetric inhibitor is by far the simplest and most conformationally rigid member of the PP inhibitor group.

Extensive SAR data have been compiled on cantharidin and its relatives even before its in vivo target was revealed to be PP1 and PP2A in 1992.[63–67] Removal of one or both methyl substituents decreases activity significantly, and adding *endo* substituents completely abolishes activity. Replacement of the bridging ether with a carbocyclic methylene significantly decreases activity as well. The most potent analogue of this series is endothall thioanhydride (didesmethyl cantharidic acid thioanhydride).[64–66] The data from direct assay of phosphatase activity show that removal of one or both methyl substituents from cantharidin decreases activity incrementally.

More than 40 analogues of cantharidin were tested in mice toxicology screens and by herbicidal activity (the commercially available herbicide endothall is *bis*-norcantharidin).[68,69] Again, although the LD_{50} data do not necessarily parallel IC_{50} trends, substantial reduction of toxicity is instructive. Substitution of the ring at positions 5 and 6 is generally deleterious, with *exo* substituents being much worse. Isomerization of the anhydride from *exo* to *endo* substantially decreases activity as does formation of cantharidin imide.

Whether cantharidin or cantharidic acid is the PP-inhibitory form remains uncertain. Curiously, two independent groups have reported that the anhydrides of cantharidin and several analogues are ~eightfold more active than their corresponding diacids in vitro.[65,67] However, their data describing the hydrolytic stability of cantharidin are contradictory with values ranging from complete hydrolysis in 40 min (1:1 DMSO-d_6/50 mM Tris–HCl buffer at pH 7.0) to ~5% hydrolysis after 24 h (4:1 DMSO-d_6/D_2O, 40 °C). It is noteworthy that cantharidin competitively binds PP2A with pyrophosphate meaning it is likely binding the PP active site.[65]

The polyketides. Arguably, the most interesting and complex structures of this class of inhibitors are the polyketides (Fig. 5). They have been the focus of considerable synthetic effort over the past 15 years, and many groups have reported total syntheses of these challenging targets.

Okadaic acid (OA), was the first of these inhibitors discovered.[70–72] It was initially isolated from the marine sponges *Halichondria okadaii* and *H. melanodocia* but later found to actually come from certain varieties of marine plankton and to accumulate in sponges and bivalves through filter feeding. OA is the causative chemical agent of diarrheic seafood poisoning, a serious illness resulting from the ingestion of contaminated shellfish. Several naturally occurring congeners of okadaic acid, known as acanthafolicin and the dinophysistoxins 1–4, have also been isolated; they are equally potent with OA and consist of slight structural modifications to the parent OA skeleton.[71]

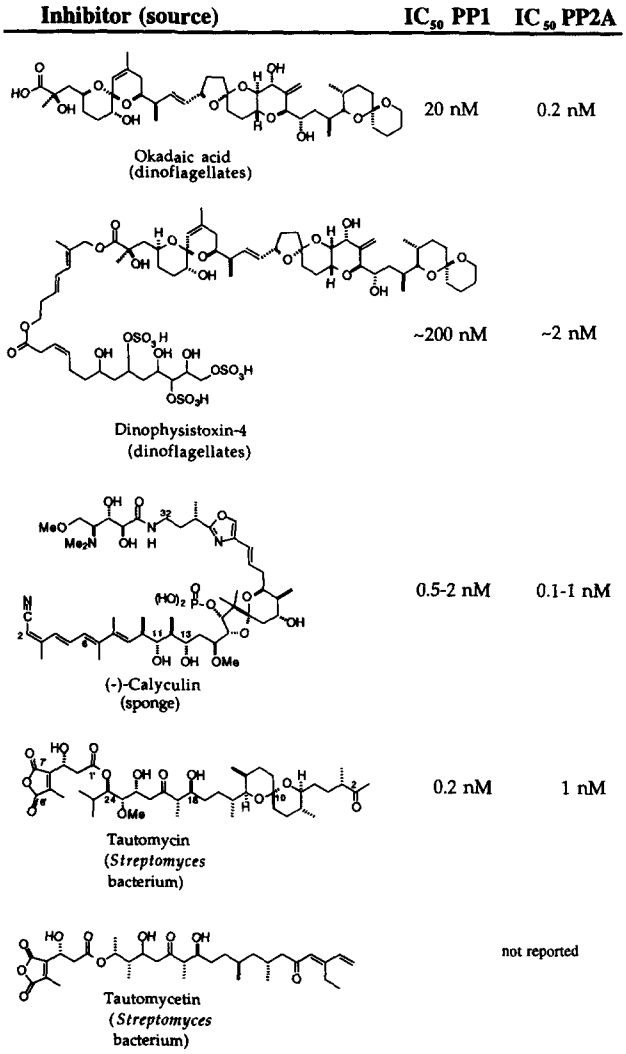


Figure 5. Known polyketide inhibitors of PP1 and PP2A.

Takai has shown that OA is a potent and selective inhibitor of PP1 and PP2A (ID_{50} = 315 and 1.2 nM, respectively), while of the remaining two major protein phosphatases, PP2B is only weakly inhibited, and PP2C is not affected.[73] In this regard, OA has become a powerful tool for the study of biological processes mediated by protein phosphorylation. Three structure–activity relationships for okadaic acid have been published.[73–75] To summarize, esterification or removal of the acid abolishes activity completely. Ozonolysis of the alkene and reduction of the resulting aldehydes gives two spiroketal portions that both inhibit dephosphorylation of phosphorylated histone H1 albeit not as effectively as OA itself. Etherification or removal of hydroxyl-7 causes negligible change, but analogously changing the 2-hydroxyl also abolishes activity. Last year, the Wright group discovered a new water-soluble variant of okadaic acid, dinophysistoxin-4 (DTX-4, see Fig. 5), that bears a sulfated polyol ester of the carboxylic acid moiety.[76] This is a curious result considering esterification of okadaic acid abolishes activity. Although this natural analogue of OA has 10–50-fold less potency than OA itself, removal of all three sulfate groups gives an analogue that still inhibits PP1 and PP2A.

Calyculins A–H were isolated from the marine sponge *Discodermia calyx*[77–80] and differ only in permutations of the C2 and C6 alkene geometries and a methyl substituent at C32. All of these congeners are equally potent for both PP1 and PP2A and of the few semisynthetic analogues that have been explored, decahydrocalyculin retains activity but the C11–C13 acetonide has an IC_{50} > 1 mM.[81]

Tautomycin (TM) was isolated from the fermentation broth of *Streptomyces spiroverticillatus* in 1987 and was structurally elucidated in 1993.[82–85] It exhibits antifungal activity, hepatotoxicity, and induces bleb formation in K562 human myeloid leukemia cells which was correlated with its potent inhibition of PP1 and PP2A.[86,87] It inhibits PP1 with an IC_{50} of 0.2 nM and PP2A with an IC_{50} of 1 nM—*the only* small molecule inhibitor that is selective for PP1 (albeit only fourfold).

Inspection of TM immediately reveals analogy to cantharidin since they both possess constrained anhydrides (maleic and succinic, respectively). In an attempt to ascertain whether the anhydride or the diacid of tautomycin is the pharmacologically active species, Sugiyama et al. showed that the diacid form of TM was stable at both pH 6.0 and 8.4 (HEPES or Tris buffers) and the anhydride was 80% hydrolyzed at pH 8.4, but only 10% hydrolyzed at pH 6.5 in vitro on the phosphatase assay timescale (~15 min). The diacid showed stronger activity (IC_{50} of 8 nM at pH 6.5 and 8.4) than the anhydride (IC_{50} of 356 nM and 63 nM at pH 6.5 and 8.4, respectively)—opposite the trend exhibited by cantharidin.[88] Also unlike cantharidin, a synthetic TM analogue in which the majority of the molecule attached to C1′ has been replaced by a methyl ester is essentially inactive.[89] The only other SAR data published for TM show that deoxygenation or β-elimination of the C22-hydroxyl substituent destroys activity.[88]

Tautomycetin is a tautomycin variant that is structurally similar, but lacks the spiroketal portion. It was isolated from *Streptomyces griseochromogenus* and induces the same morphological changes in cells as TM does, but data describing its PP-inhibition have not been published.[90–92]

All of the natural products above are collectively referred to as the Okadaic Acid-class inhibitors[27,81] since OA was the first discovered, and all of them (thyrsiferyl not reported) exhibit competitive kinetics with [^{3}H]-okadaic acid as do the endogenous protein inhibitors (NIPP, I_1^{PP2A}, and I_2^{PP2A} not reported).[86,94–98] These results imply that the natural products bind to similar (perhaps the same) region of the phosphatases.

The OA-class all inhibit PP1 and PP2A (with several members also affecting PP4 and PP5), but PP2B

Table 1. A summary of Ser/Thr-specific phosphatases and their inhibitors

	PP1	PP2A	PP2B	PP2C	PP4	PP5
Microcystin-LR	0.1 nM	0.1 nM	ND	NI	8 pM	>1 nM
Nodularin-Y	1.8 nM	0.03 nM	8.7 mM	NI	ND	ND
Okadaic acid	3 nM	0.2–1 nM	>10 mM	NI	0.2 nM	<3 nM
Calyculin	0.3–0.7 nM	0.2–1 nM	>10 mM	NI	ND	ND
Tautomycin	0.7 nM	0.7 nM	~70 mM	NI	ND	ND
Cantharidin	0.5–2 mM	0.2 mM	>1 mM	NI	ND	ND
Thyrsiferyl	>1 mM	4–16 mM	>10 mM	ND	ND	ND
I-1/DARPP	0.5–0.8 nM	NI	NI	NI	ND	ND
I-2	0.5–0.8 nM	NI	NI	NI	ND	ND
NIPP	<1 PM	NI	NI	NI	ND	ND
I-1PP2A	NI	4 nM	NI	NI	ND	ND
I-2PP2A	NI	2 nM	NI	NI	ND	ND

NI, not inhibitory; ND, not determined; data are reported as IC_{50} values.[93] Specific inhibitors of PP2B (calcineurin) are FK506 and cyclosporin. Specific inhibitors of PP2C are ATP-g-P.

(calcineurin) is only weakly inhibited, and PP2C is unaffected making this class of small molecules invaluable for biological studies. The polyketides and cantharidin in particular have received the most attention as small molecule probes since they are all cell-permeable (the cyclic peptides and endogenous inhibitors are not). It should also be noted that in addition to cantharidin, tautomycin and tautomycetin are the only OA-class inhibitors that are available in appreciable quantity because they can be produced by bacterial fermentation rather than isolated in minute quantities from natural sources.

A growing number of publications describe the use of the above inhibitors to elucidate details of intracellular signaling pathways; i.e., the effects of selective phosphatase inhibition upon normal cell function. For example, OA has helped demonstrate that atrial natriuretic peptide is dephosphorylated by PP2A—a very important finding since this peptide is a peripheral hormone that controls blood pressure in the heart but is also thought to play an important role in neurotransmission and/or neuromodulation.[99] Calyculin, OA, and microcystin are all potent tumor promoters (on mouse skin and in the liver) and have been used to help elucidate two-stage carcinogenesis via mutations in the murine c-Harvey-*ras* gene.[100] General studies of the Ser–Thr-specific PPs and their role in human carcinogenesis have also been gaining momentum.[18,81,101,102] Lastly, several groups have placed protein phosphatase 1 at center stage since PP1 and PP2C have been shown to be responsible for the normal phosphorylation state of microtubule-associated protein which promotes the assembly of tubulin into microtubules. However, when hyperphosphorylated due to some failing of PP1 (or overactivated kinases), forms paired-helical filaments—the most characteristic molecular change associated with Alzheimer's disease.[103–110]

3. Site-Directed Mutagenesis

To date, there have been a handful of attempts to modify PP1/PP2A inhibitors in order to understand which part(s) of the molecule confers activity. Although insightful, much of the existing SAR data is incomplete because it could be obtained only via chemical modification of the parent natural products or related metabolites. Several groups have taken the complementary strategy to the SAR studies using site-directed mutagenesis to explain the effect of particular residues on both catalysis/substrate binding and the inhibition by several OA-class inhibitors on PP1 mutants. The effects on catalysis are summarized in Table 2.[111]

As can be seen from the data, the E275R mutant found in the β12–β13 loop actually improves the catalytic efficacy of PP1 perhaps due to the increase of more positive charge in the vicinity of the active site. This mutation corresponds to the change in sequence from PP1 to PP2A at this site. The conservative change of

Table 2. Kinetic constants for dephosphorylation of ^{32}P-phosphorylase a by wild-type and mutant PP1[111]

PP1	Specific activity (U/mg)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}M^{-1} \times 10^{-6}$)	V_{max} (mmol/mg/min)
Wild-type	34	10.6	38.5	3.63	62.5
E275R	69	13.2	86	6.50	139.6
C127S	49	10.4	58	5.56	94
Y272F	26	5.0	14.0	2.82	22.7
D208A	8	23.3	13.3	0.57	21.6
R96A	0.08	6.9	0.09	0.013	0.2
R221S	0.05	5.8	0.02	0.003	0.03

Y272F in the same region causes minimal perturbation in catalysis. Sequential mutation of the conserved arginine 96 and 221 is quite deleterious, and aspartic acid 208 (H-bonded to R221) is less so.

The effect of PP1 mutants on the inhibitory properties of several members of the OA-class are described below.

Table 3 shows the effect of single point mutations on the inhibition by several members of the OA class. Changing cysteine 127 to a serine (a PP1 to PP2A mutation) anomalously increases the IC_{50} of microcystin-LR which does not quite contact this residue in the X-ray structure.

Changing of residues E275 and Y272 in the β12–β13 loop has a markedly different effect on different inhibitors. The E275R mutant seems to dramatically improve only inhibition by thiophospho-DARPP and slightly improve that of microcystin although this is two residues from the contact area with β12–β13. The Y272F mutant effectively removes an important hydrogen bond to microcystin and the IC_{50} increases accordingly. In a separate study[22] (data not shown), this residue was shown to be critically important for effective inhibition of PP1 by OA, TM, and calyculin since their IC_{50}s increased by two to three orders of magnitude for the Y272F mutant. A very important finding is that R221 is important for OA and calyculin binding. Curiously, thiophospho-DARPP is barely affected. R221 appears to be more important than R96 with respect to calyculin and OA. Lastly, Zhang et al. made a chimeric mutant of PP1 whereby they replaced the GEFD (residues 274–277) with the YRCG sequence of PP2A.[112] Although this mutant did not change the inhibition by tautomycin or cantharidic acid, microcystin, nodularin, and calyculin IC_{50}s were increased between four- and sevenfold but OA was *decreased* 10-fold. The last result implies that about half of OA's 100-fold preference for PP2A over PP1 comes from a more favorable interaction with the sequence in the β12–β13 loop.

4. Conclusions

Because of the ubiquitous nature of phosphoprotein signaling, there has been intense interest in the interaction between I-1/DARPP and the phosphatases; there is still much to learn, however. As mentioned previously, PP1 and PP2A have broad, overlapping substrate specificities such that most inhibitors cannot discern between them. Although okadaic acid, cantharidin, and thyrsiferyl-23-acetate are PP2A selective, inhibition of PP2A indirectly inhibits PP-1 in vivo because of the resultant buildup of I-1 or DARPP-32 (both of which are PP1 inhibitors, Fig. 2). Interestingly, there are no known natural toxins that are highly selective for PP1; the best is tautomycin, but only by a factor of 4. This makes sense from an evolutionary point of view—inhibition of PP2A knocks out two phosphatases for the metabolic price of one—but it makes dissecting PP1 and PP2A signaling pathways difficult. Although I-1 and DARPP-32 are PP1-specific inhibitors, they are not membrane permeable and require microinjection into cells where they are vulnerable to protease catabolism. Thus, it is currently not possible to effectively study the effects of shutting down PP1-dependent pathways without simultaneously doing the same to PP2A. As a result, the pathways under the control of PP1 alone cannot be readily distinguished from those of PP2A (or both). One of the ultimate goals is to discover selective inhibitors of PP1 for use as greatly improved mechanistic probes. Such inhibitors would pave the way for a much deeper understanding of how both PP1 and PP2A mediate intracellular signaling processes. Most importantly, a PP1-selective inhibitor would complement the other specific/highly selective inhibitors of PP2A, PP2B, and PP2C such that one could rapidly ascertain: (1) which of these enzymes is involved in dephosphorylation of a phosphoserine- or phosphothreonine-containing protein and (2) what control (Ca^{2+}-dependence, phosphatase activation conditions, etc.) is exerted on essentially any pathway tied to that protein. This endeavor will also provide experimental evidence for or against the hypothesis that the okadaic-class inhibitors share a common pharmacophore and whether this group of natural products mimics the active portion of the PP1-specific inhibitory phosphoproteins DARPP and I-1.

Table 3. Effects of OA-class inhibitors on wild-type and mutant PP1[111]

	IC_{50} (nM)			
PP1	**Microcystin-LR**	**Okadaic Acid**	**Calyculin-A**	**S-DARPP-32**
Wild-type	0.023	45	0.45	115
E275R	0.0091	48	0.4	2.2
C127S	0.56	78	2.2	250
Y272F	0.8	1150	48	310
D208A	4.9	>5000	>500	4.2
R96A	35	260	58	640
R221S	29	>5000	>500	960

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(*Received in U.S.A.* 21 *February* 1997; *accepted* 30 *June* 1997)

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