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MOLECULAR MECHANISMS UNDERLYING INHIBITION OF PROTEIN PHOSPHATASES BY MARINE TOXINS

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1. ABSTRACT

The protein serine/threonine phosphatases constitute a unique class of enzymes that are critical regulatory enzymes as they must counteract the activities of thousands of protein kinases in human cells. Uncontrolled inhibition of phosphatase activity by toxic inhibitors can lead to widespread catastrophic effects. Over the past decade, a number of natural product toxins have been identified which specifically and potently inhibit protein phosphatase-1 and –2A. Among these are the cyanobacteria-derived cyclic heptapeptide microcystin-LR and the polyether fatty acid okadaic acid from dinoflagellate sources.

The molecular mechanism of the potent inhibition of protein phosphatase-1 by these toxins is becoming clear through insights gathered from diverse sources. These include: 1. Comparison of structural variants of the toxins, 2. Delineating the structural differences between protein phosphatase-1 and –2A accounting for their differing sensitivity to okadaic acid, 3. Determination of the crystal structure of protein phosphatase-1 with microcystin-LR bound and, most recently, 4. Mutagenesis of protein phosphatase-1. Taken together, these data point to a common binding site on protein phosphatase-1 for okadaic acid and microcystin-LR. However, the details of these data suggest that each toxin binds to the common site in a subtly different way, relying on common structural interactions to different degrees. Finally, the insights derived from protein phosphatase-1 may help explain different sensitivities of other protein serine/threonine phosphatases to toxin inhibition due to the high degree of structural conservation among many members of this enzyme family.

2. INTRODUCTION

The protein serine/threonine phosphatases represent a unique class of enzymes in eukaryotic cells that catalyze the dephosphorylation of phosphoserine or phosphothreonine residues. Classically, these enzymes have been distinguished into four types (PP-1, PP-2A, PP-2B (calcineurin), and PP-2C) by their sensitivity to inhibitors, requirement for cations, and *in vitro* substrate specificity (1). The catalytic subunits of three of these phosphatases (PP-1c, PP-2Ac, and the A subunit of PP-2B, simply referred to as PP-2B hereafter) constitute a single gene family, termed the PPP gene family (2). However, additional protein serine/threonine phosphatase catalytic

subunits possessing PPP family structures have been recently identified, including PP-4, PP-5, PP-6, and PP-7 (3-7). This review will focus on the three traditional PPP enzymes that includes PP-1c, which shares 49% sequence identity with PP-2Ac and 40% sequence identity with PP-2B.

Regulation of the level of protein phosphorylation in cells is critical to maintaining homeostasis. The limited number of protein serine/threonine phosphatase catalytic subunits described above, in combination with a growing number of regulatory and targeting subunits (7-10), must counterbalance all of the phosphorylating activity of thousands of protein kinases believed to exist in the human genome (11). Therefore, understanding the regulation of these phosphatases is central to a full appreciation of the complexities of signal transduction mediated by reversible protein phosphorylation in eukaryotic cells.

The types of physiological processes in which the PPP gene family phosphatases play a regulatory role are diverse and not within the scope of this review to fully delineate (for reviews, see 1,12-15 and other chapters in this volume 16-19). For example, PP-1c is responsible for regulating a myriad of critical biochemical processes in eukaryotic cells, including regulation of glycogen metabolism, coordination of the cell cycle, and control of gene expression. Therefore, it is not surprising that unregulated inhibition or activation of phosphatases can have detrimental systemic effects.

Within the last decade, it has become clear that a number of natural product toxins from quite diverse sources exert their effects through direct and potent inhibition of PP-1c and PP-2Ac (20-22). The two most widely studied natural product phosphatase inhbitors are cyclic heptapeptide microcystins from both freshwater and marine cyanobacteria and okadaic acid, a polyether fatty acid first characterized as the causative agent of diarrhetic shellfish poisoning in humans. Microcystins generally inhibit both PP-1c and PP-2Ac with similar potency, usually in the nanomolar range, depending on the conditions of the assay and the structural variant being tested. Okadaic acid inhibits PP-2Ac about 10- to 100-times better than PP-1.

There are other important natural product phosphatase inhibitors including cyclic pentapeptide nodularins from mussel and cyanobacteria which are structurally related to microcystins (23-25) and exhibit similar phosphatase inhibition characteristics. Calyculin A, an octamethyl polyhydroxylated fatty acid isolated from marine sponges, is structurally unrelated to okadaic acid but possesses similar phosphatase inhibition and tumor-promoting activities (26, 27).

Still more potent natural product phosphatase inhibitors are found in diverse environments. For example, the polyketide toxin tautomycin is produced by a soil bacterium (28, 29). Other phosphatase inhibitors are produced by beetles (cantharidin; 30) and fungi (fumonisin B1; 31, 32).

What is astonishing is that while all these inhibitors possess unrelated linear chemical structures and come from very diverse origins, they all specifically and potently target the same protein phosphatases. This supports the contention that the phosphatases play extremely important physiological roles since they are important enough to be specifically targeted by natural products that have evolved over a long period of time from very distinct sources.

This review will focus on the growing body of knowledge regarding how okadaic acid and microcystins interact with PP-1c. It must be kept in mind that the high degree of structural similarity between the PPP family of protein phosphatases suggests that differences in toxin sensitivity between phosphatases may be explained through specific structural differences within a similarly folded catalytic core. Comparison of the structures of the PPP phosphatases may therefore provide insight into the structural determinants of toxin sensitivity or resistance for other phosphatases.

3. OKADAIC ACID

3.1. Diarrhetic shellfish poisoning

Over twenty years ago, a form of gastroenteritis that results from eating mussels was first identified and designated as diarrhetic shellfish poisoning (DSP) (33). Although the first scientific report of this condition has been relatively recent, it is believed that the condition now known as diarrhetic shellfish poisoning has existed for centuries in Japanese folklore where it is thought that mussels may be poisonous during the season of the paulownia flowers, which is between the months of June and July. DSP is a serious human health hazard and occurs worldwide, especially in countries where shellfish are a regular part of the diet, such as Japan. Symptoms of those suffering from DSP are mainly gastrointestinal problems such as diarrhea, nausea, vomiting, and abdominal pain. These symptoms generally are manifested quickly; within 30 minutes of consuming intoxicated mussels (34). Bouts of diarrhea and vomiting generally occur four or five times over the course of the poisoning, which lasts three days on average. However, severe cases of DSP have been documented as causing up to 20 bouts of vomiting and diarrhea a day (33).

The causative agent of DSP was isolated and characterized soon after the first report of the condition (35). The compound, termed dinophysistoxin-1, was shown to be the 35*S*-methyl variant (36) of a cytotoxic 38-carbon polyether fatty acid isolated from the marine sponges *Halichondria okadaii* and *Halichondria melanodocia*, termed okadaic acid after one of the sponges (37) (figure 1). It was observed that mussels became toxic upon feeding on the marine dinoflagellate *Dinophysis fortii*, from which the name dinophysistoxin was derived. Since other dinoflagellates, in particular the *Prorocentrum* species, were also known to be toxic (36, 38), the nature of that toxicity was investigated and it was soon discovered that *Prorocentrum lima* is a primary producer of okadaic acid and that this toxin also causes DSP (39).

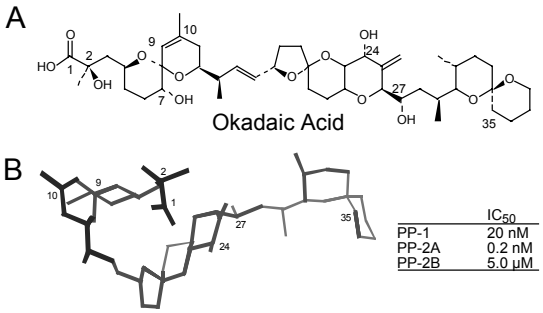

Figure 1. Okadaic acid. A**.** Linear structure of okadaic acid. B. Crystal structure of okadaic acid and IC_{50}'s for protein serine/threonine phosphatases. Selected carbon atoms are denoted by their linear structure location. Note that the carboxy group ookadaic acid bends back on itself to form a pseudo-ring structure with a hydrophobic tail reminiscent of microcystin-LR (see figure 2). IC_{50} values vary depending on conditions of the assay and are given as a general guide only.

3.2. Dinoflagellates

Dinoflagellates are a very ancient group, being first found in the fossil record about 900 million years ago (40). Because the fossil record is incomplete, however, it is likely that dinoflagellates are older, perhaps as old as the first eukaryotes which existed approximately 2 billion years ago. The initial observation that dinoflagellate DNA lacked histones and nucleosomes, mitosis occurred through a membranous mechanism, and DNA was replicated continuously, fostered the hypothesis that dinoflagellates were intermediate between prokaryotes and eukaryotes (41). As such, dinoflagellates were initially classified as mesokaryotic to distinguish their intermediate evolutionary nature.

Recently, more detailed evidence is accumulating to suggest that dinoflagellates are indeed true eukaryotes, including the demonstration that mitosis among dinoflagellates involves microtubules and includes a definite cell cycle (42). RNA sequencing data also suggests that dinoflagellates emerged rather late in eukaryotic evolution in a cluster with the ciliates (*Ciliophora*) and sporozoans (*Apicomplexa*) (43).

Work from our own laboratory has demonstrated that *Prorocentrum lima* and other *Prorocentrum* species possess both serine/threonine- and tyrosine-specific protein kinases and phosphatases much like unicellular eukaryotes (44-46), supporting the hypothesis that dinoflagellates are indeed eukaryotic organisms. Intriguingly, *Prorocentrum lima* possesses both PP-1c and PP-2Ac activity which is sensitive to inhibition by okadaic acid. This raises the question of how this unicellular organism avoids the negative effects of the okadaic acid it produces. Immunolocalization experiments with dinoflagellates have demonstrated that okadaic acid is localized in their peripheral chloroplasts and autodigestive lysosomes (PAS bodies) and may be associated with membrane lipids (47). Therefore, the hypothesis that okadaic acid is sequestered inside specific dinoflagellate cellular compartments appears to be the most viable. The physiological role of okadaic acid production in dinoflagellates is unclear, but it is possible that the toxin deters predators from consuming dinoflagellate blooms.

3.3. Okadaic acid as a potent inhibitor of protein phosphatases

In 1987 it was realized that okadaic acid potently and specifically inhibits PP-1c and PP-2Ac (48), with the IC_{50} for the toxin being two orders of magnitude lower for PP-2Ac inhibition (IC_{50} of 0.2 nM) than for PP-1c inhibition (IC_{50} of 20 nM) (49) (Note: the IC_{50} values discussed in this article are *in vitro* values and are for comparison purposes. The IC_{50} value determined experimentally varies depending on assay conditions, including enzyme concentration and substrate differences). Despite sharing similar protein folding and catalytic mechanisms, PP-2B is inhibited to a much lesser extent (IC_{50} of 5 µM). Subsequently, okadaic acid has also been shown to be a powerful tumor promoter (50, 51). Structural modification studies with okadaic acid have indicated the influence of several hydroxyl groups throughout the structure at C-2, C-7, C-24, and C-27 on the phosphatases (52), suggesting that these structural elements play important roles in the interaction of the toxin with its target.

Being hydrophobic and cell-permeable (53), the differential inhibition of PP-1c and PP-2Ac activity in intact cells by okadaic acid has made this marine toxin a valuable research tool for examining phosphatase-mediated processes and interactions *in vivo* (54, 55). Such *in vivo* work must interpreted cautiously, however, since inhibition of the primary targets of okadaic acid, PP-1c and PP-2Ac (accounting for 90% of protein serine/threonine phosphatase activity in cells), may have pleiotrophic effects (for an excellent review, see 56). For example, under certain conditions okadaic acid inhibits apoptosis in cultured cells in the short term, but not in the long term (57). Contributing to the confusing effects of okadaic acid *in vivo* is the fact that all of the physiological targets of the inhibitor have not been completely characterized. For example, some of the novel PPP family enzymes are also inhibited by okadaic acid. There may be more phosphatases that are inhibited by okadaic acid or there may be other types of enzymes that are affected by the toxin. Presently, okadaic acid is a common component of *in vitro* protein kinase assays to inhibit contaminating phosphatase activity.

4. MICROCYSTINS

4.1. Barcoo fever and cyanobacteria

Blue-green algae, also known as cyanobacteria, are not true algae but rather photosynthetic bacteria that are believed to be among the first forms of life to exist on land. As such, the biochemistry of cyanobacteria is thought to be a reflection of their early evolutionary origin. Cyanobacteria in surface water has been associated with toxic effects on wildlife and domesticated animals (58). The first scientific report of the toxic effects of cyanobacteria was in 1878 in Australia when a bloom of cyanobacteria in a freshwater lake was linked with terrible

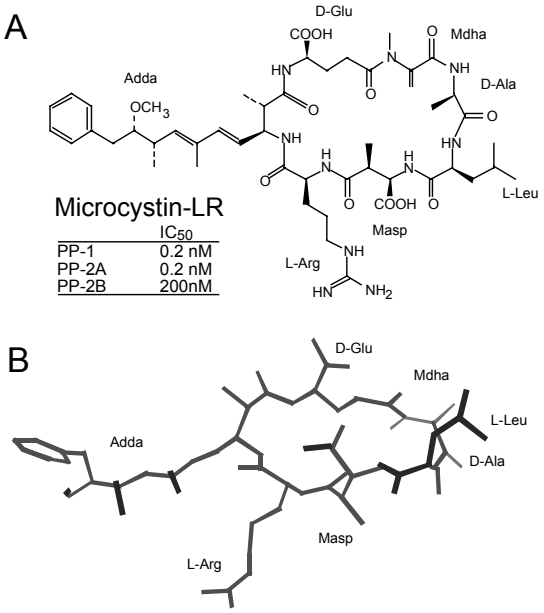

Figure 2. Microcystin-LR A. Linear structure of microcystin-LR. B. Solution structure of microcystin-LR and IC_{50} values for the phosphatases. The amino acids are labelled on each structure. Variability in the identity of L-amino acids substitutedfor L-arginine and/or L-leucine in microcystin-LR accounts for 40 known variants of microcystins, found inboth the marine and freshwater environments. Microcystins also possess the unique Adda amino acid in addition to Masp, Glu, and Mdha, as outlined in the text. IC_{50} values vary depending on conditions of the assay and are given as a general guide only.

fatalities among farm animals (59). Since that time, reports of animals being exposed to cyanobacterial toxins have been documented worldwide, with death generally being associated with severe liver damage, among other effects. Problems with human health upon exposure to water contaminated with cyanobacteria have also been reported worldwide (60). In Australia, a sickness known as Barcoo fever is now believed to be caused by ingestion of cyanobacteria. Typical symptoms include diarrhea and vomiting, which are also common effects observed among farm animals exposed to cyanobacteria. Elevated liver enzyme levels in the blood, an indicator of liver damage, have also been associated with cyanobacterial exposure (61). In February of 1996, 50 patients who underwent kidney dialysis at the Kidney Disease Institute in Caruaru, Brazil, died as a result of toxic hepatitis caused by cyanobacterial toxins in the dialysis water (62, 63). Obviously, cyanobacterial toxins are a major health concern, especially in areas of the world where surface water exists under conditions that are optimal for cyanobacterial blooms.

4.2. Microcystin structure and phosphatase inhibition

In the early 1980s, the primary structure of one of the cyanobacterial toxins that cause such drastic hepatotoxic effects was determined (64). This was a cyclic peptide containing seven amino acids, referred to as microcystin after the cyanobacteria from which it was isolated, being members of the *Microcystis* genera. This toxin is thought to enter the liver *via* bile salt transport where it is known to cause reorganization of hepatocyte cytoskeletal components (65-67), resulting in the formation of hepatic lesions. Blood seeps into the liver, enlarging it. Hepatocyte cell contents spill into the blood and, in extreme cases, hepatocytes become dislodged and freely flow in the bloodstream.

Microcystin has been characterized as a powerful tumor promoter, much like okadaic acid (68, 69). Similarly, microcystin was discovered to be a potent inhibitor of PP-1c and PP-2Ac (70-72), save that in this case the cyclic peptide inhibitor is equipotent against PP-1c and PP-2Ac, with an IC_{50} value of 0.2 nM.

Microcystin is chemically distinguished by the presence of a unique 20-carbon β-amino acid referred to as Adda ([2S, 3S, 8S, 9S]-3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6,-dienoic acid) (figure 2) (73). Microcystins differ in the nature of two variable L-amino acids indicated by suffix letters. For example, the most commonly isolated microcystin is microcystin-LR, which contains leucine and arginine in the variable amino acid positions. In addition, variations of microcystins occur as a result of the absence of methyl groups on the D-erythro-β-methyl aspartic (Masp) acid and/or the N-methyldehydroalanine (Mdha) residues. To date, over 50 distinct microcystins have been isolated and characterized (20, 21, 74).

Although microcystins have been extensively characterized in fresh and brackish water environments, they have recently been isolated in the marine environment (75), raising the likelihood that these cyclic peptide hepatotoxins contribute to marine-related illnesses. Microcystins were found to be the causative agent of netpen liver disease among Atlantic salmon farmed in the Pacific Northeast (76, 77). This disease has had devastating effects on farmed salmon and has caused the loss of millions of dollars in the fish farming industry of British Columbia and the state of Washington.

A significant biochemical difference exists between microcystins and okadaic acid. Although both rapidly bind and potently inhibit PP-1c and PP-2Ac, only microcystins form a covalent linkage with PP-1c or PP-2Ac after hours of incubation (78, 79). It has been demonstrated that microcystins form this covalent link between the Mdha residue of microcystins and Cys-273 of PP-1c, placing microcystins very close to the catalytic center of PP-1c since Tyr-272 coordinates one of the catalytic center water molecules.

5. MOLECULAR INTERACTIONS

One of the crystal structures of PP-1c determined in 1995 was a heterodimeric complex of PP-1c with microcystin-LR covalently bound to Cys-273 (80). This structure confirmed the hypothesis that microcystin-LR binds directly in the proposed catalytic center of the phosphatase (figure 3) and raised the question of the molecular mechanism of PP-1c-microcystin-LR inhibitory

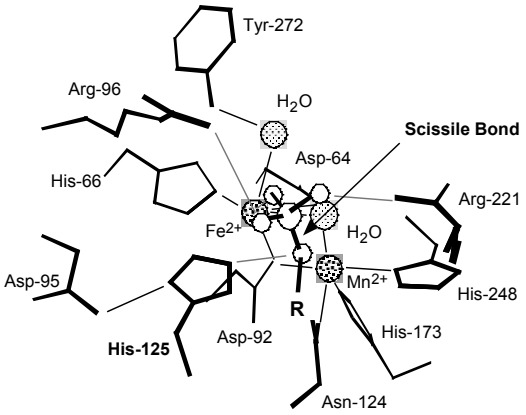

Figure 3. The binuclear catalytic center of PP-1c. Two metal ions are coordinated in the catalytic center of PP-1c by a host of residues and two water molecules, indicated by the black lines. The oxygen atoms of the target phosphate group are ligated by Arg-96, Arg 221, the Fe^{2+} of the recombinant enzyme and the catalytic His-125, indicated by the gray lines. The water molecule that bridges the two metal atoms is in-line with the phosphorus-oxygen scissile bond and acts as a nucleophile to attack the phosphorus atom. The catalytic His-125 donates a proton to the oxygen of the leaving group.

interactions. Moreover, evidence suggests that okadaic acid binds in the same site as microcystin-LR, since microcystin-LR can compete with okadaic acid for phosphatase binding sites (71, 81, 82). However, the precise nature of okadaic acid interactions with PP-1c is not known.

As with okadaic acid, microcystins have little effect upon PP-2B. This raises the question of the molecular basis for PP-2B resistance to these inhibitors in the face of PP-1c sensitivity and similar catalytic domain protein architecture (83). Thus several questions remain regarding the structural basis of toxin interaction with PPP gene family phosphatases. Microcystin-LR and okadaic acid are by far the most extensively studied marine toxins from both a biological and molecular point of view. Therefore, the molecular mechanism of interaction of microcystins and okadaic acid with PP-1c will be the focus of this review, bearing in mind the structural similarities between PP-1c and PP-2B in their catalytic cores.

Since the interaction of the marine toxins with PP-1c involves two players, past experimental strategies aimed at understanding the mechanisms of those interactions have been performed from two points of reference. One strategy has been to examine structure-function relationships between the toxins and the phosphatases from the context of the toxins. This strategy has the most historical precedent, since there are several naturally-occurring variations of microcystins and due to the ease of chemical modifications of all the marine toxins. More recently, the application of molecular biological techniques has permitted examination of the contribution of the phosphatase itself to the interaction with the marine toxins through site-directed mutagenesis and expression of recombinant enzyme. Moreover, the determination of the crystal structure of PP-1c with the marine toxin microcystin-LR bound has provided a clear structural picture of the interaction of the phosphatase with this cyclic peptide inhibitor.

5.1. Insights from structural analysis of the marine toxins

5.1.1. Okadaic acid

Several groups have analyzed the ability of different structural variations of the marine toxins on their ability to inhibit phosphatases or to exert toxic effects. Initial work with okadaic acid demonstrated that modification of the carboxylic acid group of carbon-1 (C1, see figure 1) causes significant decreases in the ability of the toxin to inhibit phosphatase activity (51, 84), such that okadaic acid with a methyl ester on C1 is completely ineffective as a phosphatase inhibitor. The same study demonstrated that methylation of the oxygen atoms associated with C2, 7, 24, and 27 also causes severe decreases in toxin effectiveness. This evidence suggests that the acid moiety of okadaic acid is of great importance to okadaic acid toxicity.

5.1.2. Microcystins

The isolation of naturally-occurring variations of microcystins has also provided clues as to the structural features of this cyclic peptide that are key to its interactions with PP-1c. Most commonly, novel microcystins were tested in a mouse bioassay to determine the LD_{50} of the toxin (50% of the lethal concentration of toxin delivered intra-peritoneally) (85, 86). The LD_{50} of microcystin-LR is approximately 50 μg/kg in mice. Since the binding and inhibition of phosphatases by microcystins form the likely basis of the effects of the toxin, LD_{50} data may provide insight into the ability of the various toxins to interact with PP-1c.

Three key points arise from these studies. First, linearization of the peptide abolishes the ability of the peptide to exert toxic effects. Secondly, modifications of the Adda sidechain have demonstrated that the overall shape of this unique amino acid is critical to microcystin interaction. Slight modifications, including demethylation of the C9 of the Adda sidechain, have little effect on microcystin toxicity. However, geometrical isomers of microcystins in which the C6 diene is changed from an extended *trans* E configuration to the *cis* Z configuration possess drastically reduced toxicity. These decreases in toxicity are reflected in 100-fold increases in the IC_{50} of the toxins toward phosphatases (69). Finally, it has been demonstrated that the free carboxylic acid on the glutamic acid residue to microcystins is important to toxicity. Any modification of this moiety results in complete loss of toxicity.

Studies with structural isomers of microcystins also demonstrate which parts of the toxin are not important to toxin-phosphatase interactions. For example, several isomers of microcystin-LR have a different amino acid in place of the variable arginine, including alanine,

Table 1. The amino acids of the L7 loops from PP-1c, PP-2Ac, PP-2B (the classical PPP family members) and PP-4, PP-5, PP-6, PP-7 (novel PPP enzymes). Shown are the amino acids surrounding the L7 loop (underlined) from these phosphatases

Phosphatase	L7 Loop
PP-1c (268-281)	SAPNYCGEFDNAGA
PP-5 (447-460)	SAPNYCDQMGNKAS
PP-7 (423-436)	SASNYYEEGSNRGA
PP-2Ac 259-272)	SAPNYCYRCGNQAA
PP-4 (258-271)	SAPNYCYRCGNVAA
PP-6 (257-270)	SAPNYCYRCGNIAS
PP-2B (307-320)	SAPNYLDVYNNKAA

methionine, phenylalanine, leucine, and homoserine, with no appreciable change in toxicity. These results are also confirmed by inhibition of PP-1c by arginine-substituted microcystins (74), which all possess IC_{50} values comparable to microcystin-LR. Small effects are seen with microcystins in which the variable leucine is changed to another amino acid. When that amino acid is another hydrophobic residue, insignificant changes occur. Only slight increases in LD_{50} are observed when a charged residue, such as arginine or methionine sulfoxide (Met(O)) is introduced in the leucine position (85).

5.2. Insights from sequence comparisons of PP-1c and PP-2Ac

Prior to the determination of the crystal structure of PP-1c, comparisons of the inhibition of PP-1c and PP-2Ac by the marine toxins provided the most insight into the interaction of the phosphatases with the toxins. As noted earlier, PP-1c and PP-2Ac are inhibited equally by microcystin-LR, while PP-2Ac is 100-fold more sensitive to okadaic acid than PP-1c. Conversely, PP-2B is relatively resistant to the marine toxins.

An initial observation that some hamster cell lines were resistant to okadaic acid toxicity lead to the finding that a single point mutation in the gene encoding PP-2Ac is responsible for this effect. This mutation, consisting of the substitution of Cys-269 in PP-2Ac with a glycine residue, causes PP-2Ac activity to be 2 to 4-times more resistant to okadaic acid in these cells (87). It was noted that this cysteine is part of a C-terminal four amino acid-motif that is variable between PP-1c and PP-2Ac. This motif lies in a turn between β-strands 12 and 13 of the PP-1c structure, also known as the L7 loop of PP-2B (83), which will be referred to as the L7 loop for all the PPP phosphatases hereafter (table 1). The production of a chimeric form of PP-1c through mutagenesis of its L7 loop such that it possessed the amino acids present in the L7 loop of PP-2Ac resulted in a mutant form of PP-1c that was 10-times more sensitive to okadaic acid than the wild type enzyme (88). In another study, mutation of amino acids within the L7 loop of PP-2Ac resulted in enzymes that were 50- to 100-times less sensitive to inhibition by okadaic acid (89). Thus, it was concluded that the L7 loop of the phosphatases must form part of an okadaic acid-binding domain and that the different amino acid composition of PP-1c and PP-2Ac in this region accounts in part for the different sensitivity of these phosphatases to the dinoflagellate-derived toxin.

These studies also shed light on the interaction of the cyclic peptide inhibitors with PP-1c and PP-2Ac. Since microcystins inhibit the binding of okadaic acid to cellular proteins (72, 81), it was suggested that these toxins bind their target proteins on the same or overlapping sites, although it was initially difficult to believe that such chemically disparate compounds could interact at a common single site. Indeed, the chimeric form of PP-1c possessing the PP-2Ac L7 loop which displayed 10-fold increases in its sensitivity to okadaic acid exhibited modest decreases in sensitivity to microcystin-LR. This strengthened the view that while the toxin-binding sites for okadaic acid and microcytsin-LR might overlap on PP-1c and PP-2Ac, they are probably not exactly the same. However, the possibility that a common site may be present made the region surrounding the L7 loop of PP-1c and PP-2Ac particularly interesting. Mutagenesis studies of the region surrounding the L7 loop of PP-1c revealed that Tyr-272 plays a crucial role in toxin binding (90). Mutation of this residue caused significant increases in toxin resistance of the phosphatase. As noted above, it has been determined that microcystins form a covalent linkage with PP-1c between the Mdha sidechain of the toxin and Cys-273 (78, 79). This covalent linkage further supports the hypothesis that the marine toxin-binding domain of PP-1c includes the L7 loop of the phosphatase, since Cys-273 joins Tyr-272 and the four amino acids identified as playing a role in okadaic acid sensitivity.

5.3. Insights from the crystal structure of microcystin-LR bound to PP-1c

With the advent of the crystal structure of PP-1c with microcystin-LR bound, precise structural interactions between the inhibitor and the phosphatase could be observed directly for the first time (80). The catalytic site of PP-1c is located at the convergence of three surface grooves, forming a Y-shape. These three grooves have been termed the hydrophobic groove, a conserved arrangement of hydrophobic sidechains also found in PP-2Ac and PP-2B, the acidic groove, containing a number of acidic sidechains, and the C-terminal groove, which is formed in part by the C-terminus of the protein. The amino acids that bind the two metal ions in the proposed binuclear metal catalytic center are found at the junction of these three grooves (91-93).

5.3.1. Interactions with catalytic metals

Microcystin-LR binds to PP-1c through interactions with three regions of the phosphatase. Microcystin-LR coordinates with the two catalytic metal atoms indirectly *via* two water molecules through the carboxyl group of the glutamic acid and adjacent carbonyl oxygen of the toxin. This accounts for the observation that the carboxyl group of the microcystin glutamic acid is absolutely necessary for toxicity by the cyclic peptide inhibitor. In addition, the carboxyl group of the Masp of microcystin-LR interacts with Arg-96 and Tyr-134 of PP-1c, thereby abrogating the involvement of Arg-96 in interaction with the phosphate of phosphatase substrates.

Thus, microcystin-LR completely blocks access to the active center of the enzyme. This is in contrast to the well-known inhibitors of PP-2B, cyclosporin and FK506, which block access of protein substrates to the active site of PP-2B when bound to their cognate receptors, but do not disrupt access of the small non-physiological substrate *p*NPP, which is still dephosphorylated by PP-2B when cyclosporin or FK506 is bound (94).

5.3.2. Interactions with the L7 loop

Cyclic peptide toxin interactions with the C-terminal groove also occur at the L7 loop of PP-1c. The crystal structure demonstrates that Tyr-272 packs closely to the leucine of microcystin-LR, suggesting that hydrophobic interactions may occur there. This explains the biochemical evidence that PP-1c sensitivity to microcystin-LR is decreased when Tyr-272 is mutated, especially when the aromatic moiety of Tyr-272 is replaced (90). Similarly, as discussed previously, replacement of the leucine of microcystin with other hydrophobic residues has little effect on toxicity, whereas substitution with charged residues causes slight decreases in toxicity (85, 86). The covalent linkage between the Mdha sidechain of the toxin and Cys-273 of PP-1c was also observed in the crystal structure, verifying what was earlier shown employing other forms of biochemical analysis.

5.3.3. Hydrophobic interactions

The third region of PP-1c with which microcystin-LR interacts is the hydrophobic groove, which accommodates the Adda sidechain of the toxin. The packing of the Adda group to the PP-1c hydrophobic groove is quite close, suggesting that a significant portion of the binding potential for the toxin is derived from hydrophobic interactions in this region. It is not surprising, therefore, that alterations in the absolute configuration of the Adda sidechain in the form of the 6(Z) geometric isomers lead to reduced potency of the toxin. This would completely alter the orientation of the Adda in the hydrophobic groove such that hydrophobic interactions are lost and thus microcystins do not bind.

In addition to explaining the biochemical evidence surrounding important interactions between microcystins and PP-1c, the crystal structure also explains the features of microcystins that are not important for phosphatase binding. As discussed earlier, the variable amino acid arginine of microcystin-LR can be substituted with a variety of different amino acids with little effect on the inhibitory properties of the toxin. The crystal structure of microcystin bound to PP-1c demonstrates that the arginine sidechain of the toxin is directed away from the catalytic site of the phosphatase and is not involved in any productive interactions with the phosphatase. Therefore, it is logical that substitutions of this amino acid would not cause any change in inhibition characteristics of the toxin.

An additional insight into the interaction of microcystins and PP-1c derived from the crystal structures is that the conformation of microcystin-LR does not change significantly from its solution structure (95), which might contribute to the high affinity of PP-1c for the toxin. Comparison of the crystal structures of PP-1c with and without microcystin-LR bound indicates that the L7 loop of PP-1c shifts slightly to avoid steric conflict between Tyr-272 and the Mdha sidechain of microcystin-LR (91). This disrupts the potential for interactions between the hydroxyl of Tyr-272 and one of the catalytic metal atoms in the active site *via* a water molecule thereby destabilizing the catalytic conformation of the enzyme. Moreover, this observation suggests that flexibility of the L7 loop of PP-1c may be important for toxin binding and inhibition of the enzyme.

5.4. Insights from mutagenesis studies

The publication of the crystal structures of PP-1c raised new questions regarding the catalytic mechanism of PP-1c and interactions between this phosphatase and microcystin-LR. Recently, several groups have investigated the contribution of various sidechains of PP-1c to catalysis through mutagenesis of important sidechains (96, 97, 99). These studies verified the importance of several proposed catalytic residues, including Arg-96 and Arg-221, which are involved in coordinating with the phosphate group of the substrate and stabilizing the pentacoordinate transition state, and Asn-124 and His-248, which coordinate one of the metal atoms in the catalytic site. In addition, mutations of His-125 lead to mutant PP-1c with no detectable activity, supporting the hypothesis that this residue may be the catalytic nucleophile.

Some of the mutant forms of PP-1c designed to test the catalytic mechanism of PP-1c were also tested for their ability to be inhibited by microcystin-LR and okadaic acid (96). Previous studies demonstrated that mutations in the acidic groove of PP-1c had no effect on marine toxin binding and inhibition (97), verifying the crystal structure data which suggests that microcystin-LR does not interact with the acidic groove.

In the case of microcystin-LR, mutation of the phosphate-coordinating Arg-96 or Arg-221 caused large increases in IC_{50} for the mutant PP-1c; in the order of 1,500-fold increases in resistance (96). These results verified the crystal structure data which suggests that Arg-96 interacts with the carboxyl group of the Masp sidechain. It is also possible that the carbonyl oxygen of the arginine sidechain of microcystin might interact with Arg-221, explaining that result. Large undetermined increases in the IC_{50} of okadaic acid were also observed for mutations of the two arginine residues (96). These increases in toxin resistance were not quantified because of the large concentrations of okadaic acid required to test the mutants. Therefore, it appears that the two phosphate-coordinating arginine residues are also important for okadaic acid interaction with PP-1c.

Significant increases in microcystin resistance were observed for mutations of Asn-124 and His-248 (96, 97). These amino acids are located at the junction of the hydrophobic groove of PP-1c with the catalytic site, suggesting that modification of these positions might disrupt the binding of the Adda sidechain of microcystins, which is an important structure for toxin sensitivity.

Similar increases in resistance were seen for these mutants when okadaic acid was employed as the inhibitor.

It has been hypothesized that hydrophobic interactions may be a significant factor in the tight-binding affinity of the marine toxins for PP-1c (98). Our laboratory has therefore examined the role of hydrophobic interactions between the Adda sidechain of microcytsin-LR and the hydophobic groove of PP-1c in marine toxin potency. A major player in this region appears to be Trp-206, which lies within 4 Å of microcystin's Adda sidechain in the bound crystal structure. When Trp-206 is substituted with an alanine residue, the resulting mutant PP-1c possesses severely compromised specific activity, suggesting that hydrophobic interactions may be important for catalysis. Moreover, the W206A mutant possesses increased resistance to the marine toxins, with IC_{50} values being 20,000-fold greater for microcystin-LR and 5,000-fold greater for okadaic acid (99).

The crystal structure of microcystin-LR bound to PP-1c suggests that the hydroxyl group of Tyr-134 interacts with the cyclic peptide inhibitor through a hydrogen bond. It was decided to examine the effect of removing the hypothesized hydrogen bond interaction with the toxins by generating the Y134F mutant of PP-1c. To assess the role of hydrophobic interactions involving Tyr-134, the Y134A mutant was also constructed. Mutation of Tyr-134 of PP-1c resulted in enzymes with lower specific activity than the wild type (99). This supports the hypothesis that Tyr-134 is involved in the recognition of phosphoSer and phosphoThr in PP-1c substrates (80).

The dose response curves of the Y134F mutant demonstrate that removal of the hydroxyl of Tyr-134 causes an approximately 10-fold increase in the IC_{50} for the mutant when microcystin-LR is tested, supporting the hypothesis that a hydrogen bond is present between the inhibitors and PP-1c. The dose response curve of the Y134F mutant for okadaic acid demonstrates no significant change in IC_{50}. This suggests that a hydrogen bond does not occur between PP-1c and bound okadaic acid. This data suggests that, although the marine toxins appear to bind to the same region of PP-1c, they do so through subtly different interactions. For example, the cyclic peptide inhibitors utilize hydrogen bonding with Tyr-134 of PP-1c while okadaic acid does not.

Analysis of the importance of hydrophobic interactions between the inhibitors and Tyr-134 was achieved by the construction of the Y134A PP-1c mutant. In this case, the IC_{50} of okadaic acid increases approximately 1,500-fold. The IC_{50} of microcystin-LR also increases, being about 600-fold greater (99). These dose response curves demonstrate that, once again, the different marine toxins may employ similar interactions.

6. MOLECULAR MODELING OF OKADAIC ACID BOUND TO PP-1C: A COMMON MARINE TOXIN BINDING SITE

Taken together, all of the data presented here suggest that the marine toxins interact with several residues in and about the proposed active site of PP-1c, in addition to the L7 loop. Moreover, the evidence suggests that okadaic acid binds to the same site as microcystin-LR. Despite their dissimilar linear chemical structures, early molecular modeling studies indicated that the cyclic peptide inhibitors possess a similar overall shape, with the carboxyl group of okadaic acid occupying a position similar to that of the carboxyl group of the Glu of microcystin (100). This hypothesis is supported by biochemical evidence that demonstrates that modification of either the carboxyl group of okadaic acid or the carboxyl group of microcystin-LR abrogates inhibition of phosphatase activity by the marine toxin.

Recently, comparisons of the crystal structure of microcystin-LR when bound to PP-1c and the structure of free okadaic acid have been performed (99, 101). These studies provided evidence that the three-dimensional structures of okadaic acid and microcystins overlap remarkably well, in contrast to their dissimilar linear chemical structures. Importantly, these studies suggested that other natural product inhibitors of phosphatases, including nodularins and calyculin A, also possess similar three-dimensional structures indicating that these specific and potent inhibitors of PP-1c and PP-2Ac are structural mimics of each other. More specifically, these studies indicated that the hydrophobic end of okadaic acid overlaps well with the hydrophobic Adda sidechain of microcystin-LR. Similarly, the C1 carboxyl group of okadaic acid also coincides with the carboxyl group of the glutamic acid sidechain of microcystin-LR.

Several groups have docked the structure of okadaic acid onto the crystal structure of PP-1c and analyzed the toxin-phosphatase interactions (99, 101, 102). These molecular models predict that the hydrophobic region of okadaic acid fits into the hydrophobic groove occupied by the Adda sidechain of microcystin-LR. Mutation of Trp-206 of the hydrophobic groove of PP-1c to an alanine residue results in profound increases in PP-1c resistance to okadaic acid, suggesting that hydrophobic interactions implied by the models are important for high-affinity binding of okadaic acid to PP-1c, just as they are for binding of microcystin-LR.

In addition, the C1 carboxyl group of okadaic acid appears to be near Arg-96 and Tyr-272 of PP-1c, similar to the carboxyl group of glutamic acid and the adjacent carbonyl from Masp on microcystin-LR. Thus, the docked okadaic acid models are supported by biochemical evidence discussed above which demonstrated that modification of the C1 carboxyl group of okadaic acid results in drastic loss of phosphatase inhibition.

The models also suggest that the C13 methyl of okadaic acid is in contact with Phe-267 of the L7 loop of PP-1c through a hydrophobic interaction. This interaction is supported by biochemical analysis of a mutant of PP-1c in which the Phe-267 position was occupied by a tyrosine residue (98, see section 7 below). This mutant exhibited no difference in inhibitor sensitivity with the wild type enzyme.

The docked okadaic acid models are also supported by analysis of the involvement of Tyr-134 in toxin inhibition of PP-1c. The models suggest that the C29 methyl group of okadaic acid is in close proximity to Tyr-134. The finding that mutation of Tyr-134 to a phenylalanine results in no significant change in toxin sensitivity supports the molecular model which suggests that a hydrogen bond between the hydroxyl group of Tyr-134 and okadaic acid does not exist. However, a larger increase in okadaic acid resistance with respect to microcystin-LR resistance was observed when Tyr-134 was mutated to alanine, suggesting that toxin inhibitor hydrophobic interactions with Tyr-134 play a greater role in the inhibition of PP-1c by okadaic acid. Overall, the model suggests that okadaic acid and microcystin-LR bind to the same region of PP-1c employing many of the same interactions, but that these two classes of marine toxins may employ subtly different mechanisms.

7. COMPARISON WITH PP-2B (CALCINEURIN) AND NOVEL PHOSPHATASES

The catalytic cores of all the PPP enzymes, including the recently characterized PP-4, PP-5, PP-6, and PP-7, possess a high degree of sequence identity. This suggests that the PPP family of phosphatases share similar protein folding and catalytic mechanisms. This early hypothesis was borne out by comparison of the crystal structures of PP-1c and PP-2B (80, 83), which demonstrate that all the amino acids believed to be critical for catalysis in PP-1c are conserved structurally in the catalytic A subunit of PP-2B. Similarly, all of the critical catalytic amino acids in PP-1c are conserved in PP-4, PP-5, PP-6, and PP-7.

Some of the amino acids important for catalysis and involved in interactions with the marine toxins, including Arg-96 and Tyr-272 of PP-1c, are absolutely conserved among the PPP family members. This level of structural conservation among the PPP enzymes might suggest that they should all be potently inhibited by the marine toxins, but this is not the case. The catalytic subunit of PP-2B is relatively resistant to the marine toxins. Comparison of the crystal structures of PP-1c and PP-2B A subunit suggests that the toxin-binding pocket of PP-2B is obstructed by a radically different conformation of its L7 loop.

A chimera of PP-1c was produced in which the entire L7 loop of PP-1c was replaced with the L7 loop of PP-2B (99). No significant difference in inhibition by okadaic acid or microcystin-LR between chimeric PP-1c and wild type PP-1c was observed. This suggested that the different L7 loop conformation of PP-2B was not inherent to the amino acids contained therein and that the loop must be influenced by structures outside the catalytic core of the enzyme. Closer examination of the crystal structure of PP-2B A subunit revealed the possibility of such an interaction between Asp-313 of the L7 loop and His-339 of its extended C-terminal tail. Since the C-terminal tail of PP-2B is unique to this PPP enzyme, the chimeric PP-1c lacked this putative interaction and it is possible that its L7 loop was free to adopt a more PP-1-like conformation which permits toxin interactions. It is hypothesized that this interaction outside the catalytic center of PP-2B is responsible for its relative resistance to the marine toxins (99). Work presently underway aims to test this hypothesis.

The sensitivity of some of the novel PPP members to inhibition by the marine toxins have been examined. PP-4 was found to possess toxin sensitivity very similar to that of PP-2Ac (3). Since it has been shown that the L7 loop of the PPP phosphatases can influence sensitivity to the marine toxins (88), it is interesting to note that PP-4 possesses an L7 loop sequence identical to that of PP-2Ac (table 1). PP-6 also possesses an L7 loop sequence identical to that PP-2Ac, but its sensitivity to the toxin inhibitors has not been determined (5). However, based on its sequence similarity to PP-2Ac in its L7 loop, it might be predicted that PP-6 will have inhibition characteristics much like PP-2Ac.

PP-5 was shown to be more PP-1c-like in its sensitivity to okadaic acid, while it was less sensitive to microcystin-LR than either PP-1c or PP-2Ac (4). Its L7 loop sequence is unique, although it does possess a cysteine as its first L7 residue, suggesting that this phosphatase might also form a time-dependent covalent bond with microcystin-LR like PP-1c and PP-2Ac (78, 79). The newly characterized PP-7 possesses a unique L7 loop with no initial cysteine residue. It is not inhibited by levels of marine toxins that inhibit other PPP enzymes (6). The mechanism for this insensitivity is unknown, but, like PP-2B, interactions within the molecule between the catalytic core and peripheral structures may be responsible. One such candidate may be the EF-hand domains located in the C-terminal tail of PP-7.

8. PERSPECTIVE

Since the release of the crystal structures of PP-1c and PP-2B in 1995, our understanding of the molecular mechanism of the inhibition of the protein serine/threonine phosphatases has increased dramatically. Based on earlier studies examining the structure-function relationships between different forms of microcystins and modified okadaic acid, more recent work based on the crystallographic information and employing molecular biological techniques has allowed us to explore the interactions between phosphatase and inhibitor on the molecular level. Presently, this body of knowledge suggests that the cyclic peptide inhibitor, microcystin-LR, and polyether fatty acid okadaic acid interact with the same subset of amino acids on PP-1c, but do so through subtly different mechanisms.

Comparison of the structures of PP-1c and PP-2B have suggested that PP-2B possesses a cryptic marine toxin binding site comprised of amino acids that are important for interaction with the toxins. The molecular mechanism of PP-2B resistance to the marine toxins will be an important area of future study. Since PP-2Ac shares a higher degree of similarity with PP-1c than PP-2B (49% in

its catalytic core) and is as sensitive to inhibition by microcystin-LR (and is more sensitive to inhibition by okadaic acid) as PP-1c, it is logical to hypothesize that interactions between PP-2Ac and the marine toxins will be similar to those with PP-1c. Recent modeling of PP-2Ac based on the crystal structure of PP-1c has been performed (102). Several of the toxic phosphatase inhibitors were docked onto this structure and possible differences between the binding modes of PP-1c and PP-2Ac were identified. Data regarding the synthesis of specific microcystin structural analogs aimed at testing the validity of some of these putative phosphatase structural differences have lent biochemical support to the PP-2Ac model employed (103). However, since a satisfactory recombinant expression system for PP-2Ac has yet to be developed, it remains a challenge to examine the direct contribution of PP-2Ac residues to toxin interactions. We can console ourselves with the growing body of work concerning PP-1c described here that points to a clearer understanding of the molecular mechanisms of marine toxin inhibition of protein phosphatases.

With a clearer understanding of the molecular mechanisms underlying the specific and potent inhibition of protein phosphatases by toxic inhibitors, it will be possible to engineer novel inhibitors that can target a specific phosphatase. For example, the synthetic microcystin analogs discussed above were shown to be more selective toward PP-1c (103). Such compounds will undoubtedly be invaluable for both *in vitro* and *in vivo* work, as it will be possible to examine the effects of a specific phosphatase, rather than inhibiting several phosphatases to variable degrees. Developing such reagents will rely on the complete characterization of all phosphatases contained in the human genome to fully comprehend the structural differences that may permit the development of phosphatase-specific inhibitors.

In addition, the work outlined herein may be applied to battling the problem of toxins present in freshwater supplies through the development of simple and sensitive phosphatase-based assays for the detection of microcystins and other phosphatase inhibitors. These sensitive assays may save millions of dollars each year in livestock losses worldwide and help improve the quality of life for people who live in regions where contaminated surface water is consumed. Such detection systems may also be applied to marine toxin hazards such as the screening of mussels for dangerous levels of okadaic acid which might lead to incidents of DSP. Alternatively, a simple detection assay could be employed in aquaculture to monitor the levels of toxins in water on site and reduce losses to netpen liver disease.

The protein serine/threonine phosphatases of the PPP family are involved in critical biological processes. Understanding how they are regulated by potent natural toxins will continue to be important as our knowledge of the complexities of phosphatase-mediated signal transduction grows. The economic and human health benefits from this fundamental research demonstrate the need to continue to expand our understanding of how different toxins from diverse sources target and inhibit the PPP family of phosphatases.

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10. REFERENCES

1. Cohen, P.: The structure and regulation of protein phosphatases. Annu Rev Biochem 59, 453-508 (1989)
2. Cohen, P. T. W.: Nomenclature and chromosomal localization of human protein serine/threonine phosphatase genes. Adv Prot Phosphatases 8, 371-376 (1994)
3. Brewis, N. D., A. J. Street, A. R. Prescott & P. T. W. Cohen: PPX, a novel protein serine/threonine phosphatase localized to centrosomes. EMBO J 12, 987-996 (1993)
4. Chen, M. X., A. E. McPartlin, L. Brown, Y. H. Chen, H. M. Barker & P. T. W. Cohen: A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus. EMBO J 13, 4278-4290 (1994)
5. Bastians, H. & H. Ponstingl: The novel human protein serine/threonine phosphatase 6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1, which are involved in cell cycle regulation. J Cell Sci 109, 2865-2874 (1996)
6. Xizhong, H. & R. E. Honkanen: Molecular cloning, expression, and characterization of a novel human serine/threonine protein phosphatase, PP7, that is homologous to Drosophila retinal degeneration C gene product (rdgC). J Biol Chem 273, 1462-1468 (1998)
7. Hubbard, M. J., & P. Cohen: Targeting subunits for protein phosphatases. Methods Enzymol 201, 414-427 (1991)
8. Hubbard, M. J., & P. Cohen: On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem Sci 18, 172-177 (1993)
9. Faux, M. C., & J. D. Scott: More on target with protein phosphorylation: conferring specificity by location. Trends Biochem Sci 21, 312-315 (1996)
10. Hartshorne, D. J. & K. Hirano: Interactions of protein phosphatase type 1, with a focus on myosin phosphatase. Mol Cell Biochem 190, 79-84 (1999)
11. Hunter, T. & G. D. Plowman: The protein kinases of budding yeast: six score and more. Trends Biochem Sci 22, 18-22 (1997)
12. Cohen, P. & P. T. W. Cohen: Protein phosphatases come of age. J Biol Chem 264, 21435-21438 (1989)
13. Bollen, M. & W. Stalmans: The structure, role, and regulation of type 1 protein phosphatases. Crit Rev Biochem Mol Biol 27, 227-281 (1992)
14. Mumby, M. C. & G. Walter: Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. Phys Rev 73, 673-699 (1993)
15. Ragolia, L. & N. Begum: Protein phosphatase-1 and insulin action. Mol Cell Biochem 182, 49-58 (1998)
16. Oliver, C. J. & S. Shenolikar: Physiologic importance of protein phosphatase inhibitors. Front Biosci 3, d961-972 (1998)
17. Rubin, E., S. Tamrakar & J. W. Ludlow: Protein phosphatase type 1, the product of the retinoblastoma

susceptibility gene, and cell cycle control. Front Biosci 3, d1209-1219 (1998)
18. Berndt, N.: Protein dephosphorylation and the intracellular control of the cell number. Front Biosci 4, d22-42 (1999)
19. Lee, E. Y. C., L. Zhang, S. Zhao, Q. Wei, J. Zhang, Z. Q. Qi & E. R. Belmonte: Phosphorylase phosphatase: new horizons for an old enzyme. Front Biosci 4, d270-85 (1999)
20. Holmes, C. F. B. & M. P. Boland: Inhibitors of protein phosphatase-1 and -2A; two of the major serine/threonine protein phosphatases involved in cellular regulation. Curr Opin Struct Biol 3, 934-943 (1993)
21. MacKintosh, C. & R. W. MacKintosh: Inhibitors of protein kinases and phosphatases. Trends Biochem Sci 19, 444-448 (1994)
22. Sheppeck, J. E., C.-M. Gauss & A. R. Chamberlain: Inhibition of the Ser-Thr phosphatases PP1 and PP2A by naturally occurring toxins. Bioorg Med Chem 5, 1739-1750 (1997)
23. Rinehart, K. L., K. I. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley & A. M. Dahlem: Nodularin, microcystin and the configuration of Adda. J Am Chem Soc 110, 8557-8558 (1988)
24. Yoshizawa, S., R. Matsushima, M. F. Watanabe, K. I. Harada, K. Ichihara, W. W. Carmichael & H. Fujiki: Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. J Cancer Res Clin Oncol 116, 609-614 (1990)
25. de Silva, E. D., D. E. Williams, R. J. Andersen, H. Klix, C. F. B. Holmes & T. M. Allen: Motuporin, a potent protein phosphatase inhibitor isolated from the Papua New Guinea sponge Theonella swinhoei Gray. Tetrahedron Lett 33, 1561-1564 (1992)
26. Ishihara, H., B. L. Martin, D. L. Brautigan, H. Karaki, H. Ozaki, Y. Kato, N. Fusetani, S. Watabe, K. Hashimoto, D. Uemura & D. J. Hartshorne: Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun 159, 871-877 (1989)
27. Suganuma, M., H. Fujiki, H. Furuya-Suguri, S. Yoshizawa, S. Yasumoto, Y. Kato, N. Fusetani & T. Sugimura: Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. Cancer Res 50, 3521-3525 (1990)
28. Cheng, X-C., T. Kihara, H. Kusakabe, J. Magae, Y. Kobayashi, R-P. Fang, Z-F. Ni, Y-C. Shen , K. Ko, I. Yamaguchi & K. Isono: A new antibiotic, tautomycin. J Antibiot (Tokyo) 40, 907-909 (1987)
29. MacKintosh, C. & S. Klumpp: Tautomycin from the bacterium Streptomyces verticillatus. Another potent and specific inhibitor of protein phosphatases 1 and 2A. FEBS Lett 277, 137-140 (1990)
30. Honkanen, R. E.: Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. FEBS Lett 330, 283-286 (1993)
31. Fukuda, H., H. Shima, R. F. Vesonder, H. Tokuda, H. Nishino, S. Katoh, S. Tamura, T. Sugimura & M. Nagao: Inhibition of protein serine/threonine phosphatases by fumonisin B1, a mycotoxin. Biochem Biophys Res Commun 220, 160-165 (1996)
32. Gelderblom, W. C., K. Jaskiewicz, W. F. Marasas, P. G. Thiel, R. M. Horak, R. Vleggaar & N. P. Kriek: Fumonisins--novel mycotoxins with cancer-promoting activity produced by Fusarium moniliforme. Appl Env Microbiol 54, 1806-1811 (1988)
33. Yasumoto, T., Y. Oshima & M. Yamaguchi: Occurrence of a new type of shellfish poisoning in the Tohoku district. Bull Japan Soc Sci Fish 44, 1249-1255 (1978)
34. Scoging, A. & M. Bahl: Diarrhetic shellfish poisoning in the UK. Lancet, 352, 117 (1998)
35. Yasumoto, T., Y. Oshima, Y. Murakami, I. Nakajima, R. Bagnis & Y. Fukuyo: Toxicity of benthic dinoflagellates found in coral reef. Bull Japan Soc Sci Fish 46, 327-331 (1980)
36. Murata, M., M. Shimatani, H. Sugitani, Y. Oshima & T. Yasumoto: Isolation and elucidation of the causative toxin of the diarrhetic shellfish poisoning. Bull Japan Soc Sci Fish 48, 549-552 (1982)
37. Tachibana, K., P. J. Scheuer, Y. Tsukitani, H. Kikuchi, D. Van Engen, J. Clardy, Y. Gopichand & F. J. Schmitz: Okadaic acid, a cytotoxic polyether from two marine sponges of the genus Halichondria. J Am Chem Soc 103, 2469-2471 (1981)
38. Nakajima, I., Y. Oshima & T. Yasumoto: Toxicity of benthic dinoflagellates in Okinawa. Bull Japan Soc Sci Fish 47, 1029-1033 (1981)
39. Murakami, Y., Y. Oshima & T. Yasumoto: Identification of okadaic acid as a toxic component of a marine dinoflagellate Prorocentrum lima. Bull Japan Soc Sci Fish 48, 69-72 (1982)
40. Bujak, J. P. & G. L. Williams: The evolution of dinoflagellates. Can J Bot 59, 2077-2087 (1981)
41. Rizzo, P. J.: The enigma of the dinoflagellate chromosome. J Protozool 38, 246-252 (1991)
42. Raikov, I. B.: The dinoflagellate nucleus and chromosomes: Mesokaryote concept reconsidered. Act Protozool 34, 239-247 (1995)
43. Cavalier-Smith, T.: Kingdom protozoa and its 18 phyla. Microbiol Rev 57, 953-994 (1993)
44. Dawson, J. F., H. L. Ostergaard, H. Klix, M. P. Boland & C. F. B. Holmes: Evidence for phosphotyrosine signaling in the okadaic acid-producing marine dinoflagellate Prorocentrum lima. J Euk Microbiol 44, 89-95 (1997)
45. Dawson, J. F., H. E. Wang & C. F. B. Holmes: Identification and characterization of cAMP-dependent protein kinase and its possible direct interactions with protein phosphatase-1 in marine dinoflagellates. Biochem Cell Biol 74, 559-567 (1996)
46. Boland, M. P., F. J. R. Taylor & C. F. B. Holmes: Identification and characterisation of a type-1 protein phosphatase from the okadaic acid producing marine dinoflagellate Prorocentrum lima. FEBS Lett 334, 13-17 (1993)
47. Zhou, J. & L. Fritz: Okadaic acid antibody localizes to chloroplasts in the DSP-producing dinoflagellates Prorocentrum lima and Prorocentrum maculosum. Phycologia 33, 455-461 (1994)
48. Takai, A., C. Bialojan, M. Troschka & J. C. Rüegg: Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. FEBS Lett 217, 81-84 (1987)
49. Bialojan, C. & A. Takai: Inhibitory effects of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem J 256, 283-290 (1988)
50. Herschman, H. R., R. W. Lim, D. W. Brankow & H. Fujiki: The tumor promoters 12-O-tetradecanoylphorbol-12-acetate and okadaic acid differ in toxicity, mitogenic

activity and induction of gene expression. Carcinogenesis 10, 1495-1494 (1989)
51. Suganuma, M., H. Fujiki, H. Suguri, S. Yoshizawa, M. Hirota, M. Nakayasu, M. Ojika, K. Wakamatsu, K. Yamada & T. Sugimura: Okadaic acid: An additional non-phorbal-12-tetradecanoate-13-acetate-type tumor promoter. Proc Natl Acad Sci USA 85, 1768-1771 (1988)
52. Nishiwaki, S., H. Fujiki, M. Suganuma, H. Furuya-Suguri, R. Matsushima, Y. Iida, M. Ojika, K. Yamada, D. Uemura, T. Yasumoto, F. J. Schmitz & T. Sugimura: Structure-activity relationship within a series of okadaic acid derivatives. Carcinogenesis 11, 1837-1841 (1990)
53. Nam, Y. K., M. Hiro, S. Kimura, H. Fujiki & Y. Imanishi: Permeability of a non-TPA-type tumor promoter, okadaic acid, through lipid bilayer membrane. Carcinogenesis 11, 1171-1174 (1990)
54. Cohen, P., C. F. B. Holmes & Y. Tsukitani: Okadaic acid: a new probe for studying cellular regulation. Trends Biochem Sci 15, 98-102 (1990)
55. Schönthal, A.: Okadaic acid – a valuable new tool for the study of signal transduction and cell cycle regulation? New Biol 4, 16-21 (1992)
56. Schönthal, A.: Role of PP2A in intracellular signal transduction pathways. Front Biosci 3, d1262-1273 (1998)
57. Morana, S. J., C. M. Wolf, J. Li, J. E. Reynolds, M. K. Brown & A. Eastman: The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. J Biol Chem 271, 18263-18271 (1996)
58. Galey, F. D., V. R. Beasley, W. W. Carmichael, G. Kleppe, S. B. Hooser, & W. M. Haschek: Blue-green algae (Microcystis aeruginosa) hepatotoxicosis in daily cows. Am J Vet Res 48, 1415-1420 (1987)
59. Francis, G.: Poisonous Australian lake. Nature 18, 11-12 (1878)
60. Carmichael, W. W.: The toxins of cyanobacteria. Sci Am 270, 78-86 (1994)
61. Lambert, T. W., C. F. B. Holmes & S. E. Hrudey: Microcystin class of toxins: health effects and safety of drinking water supplies. Environ Rev 2, 167-186 (1994)
62. Jochimsen, E. M., W. W. Carmichael, J. S. An, D. M. Cardo, S. T. Cookson, C. E. Holmes, M. B. Antunes, D. A. de Melo Filho, T. M. Lyra, V. S. Barreto, S. M. Azevedo, & W. R. Jarvis: Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. New Engl J Med 338, 873-8 (1998)
63. Pouria, S., A. de Andrade, J. Barbosa, R. L. Cavalcanti, V. T. S. Barreto, C. J. Ward, W. Preiser, G. K. Poon, G. H. Neild & G. A. Codd: Fatal microcytsin intoxication in haemodialysis unit in Caruara, Brazil. Lancet 352, 21-26 (1998)
64. Botes, D. P., A. A. Tuinman, P. L Wessels, C. C. Viljoen, H. Kruger, D. H. Williams, S. Santikarn, R. J. Smith & S. J. Hammond: The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium Microcystis aeruginosa. J Chem Soc Perkin Trans 1, 2311-2318 (1984)
65. Eriksson, J. E., G. I. L. Paatero, J. A. O. Meriluoto, G. A. Codd, G. E. N. Kass, P. Nicotera & S. Orrenius: Rapid microfilament reorganization induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. Exp Cell Res 185, 86-100 (1989)
66. Eriksson, J. E., D. Toivola, J. A. O. Meriluoto, H. Karaki, Y-G. Han & D. Hartshorne: Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. Biochem Biophys Res Commun 173, 1347-1353 (1990)
67. Hamm-Alvarez, S. F., W. Xinhua, N. Berndt & M. Runnegar: Protein phosphatases independently regulate vesicle movment and microtubule subpopulations in hepatocytes. Am J Physiol 271, C929-943 (1996)
68. Matsushima, R. N., T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W. W. Carmichael & H. Fujiki: Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol 118, 420-424 (1992)
69. Nishiwaki-Matsushima, R., S. Nishiwaka, T. Ohta, S. Yoshizawa, M. Suganuma, K. Harada, M. F. Watanabe & H. Fujiki: Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatases. Jpn J Cancer Res 82, 993-996 (1992)
70. Honkanen, R. E., J. Zwiller, R. E. Moore, S. L. Daily, B. S. Khatra, M. Dukelow & A. L. Boynton: Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. J Biol Chem 265, 19401-19404 (1990)
71. MacKintosh, C., K. A. Beattie, S. Klumpp, P. Cohen & G. A. Codd: Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett 264, 187-192 (1990)
72. Yoshizawa, S., R. Matsushima, M. F. Watanabe, K. I. Harada, K. Ichihara, W. W. Carmichael & H. Fujiki: Inhibition of protein phosphatases by microcystins and nodularin associated with hepatotoxicity. J Cancer Res Clin Oncol 116, 609-614 (1990)
73. Rinehart, K. L., K. I. Harada, M. Namikoshi, C. Chen, C. A. Harvis, M. H. G. Munro, J. W. Blunt, P. E. Mulligan, V. R. Beasley & A. M. Dahlem: Nodularin, microcystin and the configuration of Adda. J Am Chem Soc 110, 8557-8558 (1988)
74. Craig, M., T. L. McCready, H. A. Luu, M. A. Smillie, P. Dubord & C. F. B. Holmes: Identification and characterization of hydrophobic microcystins in Canadian freshwater cyanobacteria. Toxicon 31, 1541-1549 (1993)
75. Chen, D. Z. X., M. P. Boland, M. A. Smillie, H. Klix, C. Ptak, R. J. Andersen & C. F. B. Holmes: Identification of protein phosphatase inhibitors of the microcystin class in the marine environment. Toxicon 31, 1407-1414 (1993)
76. Andersen, R. J., H. A. Luu, D. Z. X. Chen, C. F. B. Holmes, M. L. Kent, M. Le Blanc, F. J. R. Taylor & D. E. Williams: Chemical and biological evidence links microcystins to salmon 'netpen liver disease'. Toxicon 31, 1315-1323 (1993)
77. Williams, D. E., T. L. McCready, M. Craig, S. C. Dawe, M. L. Kent, C. F. B. Holmes & R. J. Andersen: Evidence for a covalently bound form of microcystin-LR in salmon liver and dungeness crab larvae. Chem Res Toxicol 10, 463-469 (1997)
78. Craig, M., H. A. Luu, T. L. McCready, D. E. Williams, R. J. Andersen & C. F. B. Holmes: Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. Biochem Cell Biol 74, 569-578 (1996)
79. MacKintosh, R. W., K. N. Dalby, D. G. Campbell, P. T. W. Cohen, P. Cohen & C. MacKintosh: The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. FEBS Lett 371, 236-240 (1995)

80. Goldberg, J., H. Huang, Y. Kwon, P. Greengard, A. C. Nairn & J. Kuriyan: Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376, 745-753 (1995)
81. Matsushima, R., S. Yoshizawa, M. F. Watanabe, K. Harada, F. Mitsuru, F., W. W. Carmichael & H. Fujiki: In vitro and in vivo effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin fibroblasts. Biochem Biophys Res Commun 171, 867-874 (1990)
82. Takai, A., K. Sasaki, H. Nagai, G. Mieskes, M. Isobe, K. Isono & T. Yasumoto: Inhibition of specific binding of okadaic acid to protein phosphatase 2A by microcystin-LR, calyculin-A and tautomycin: method of analysis of interactions of tight-binding ligands with target protein. Biochem J 306, 657-665 (1995)
83. Griffith, J. P., J. L. Kim, E. E. Kim, M. D. Sintchak, J. A. Thomson, M. T. Fitzgibbon, M. A. Fleming, P. R. Caron, K. Hsiao & M. A. Navia: X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. Cell 82, 507-522 (1995)
84. Holmes, C. F. B., H. A. Luu, F. Carrier & F. J. Schmitz: Inhibition of protein phosphatases-1 and -2A with acanthifolicin. FEBS Lett 270, 216-218 (1990)
85. Rinehart, K. L., M. Namikoshi & B. W. Choi: Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). J App Phycol 6, 159-176 (1994)
86. Stotts, R. R., M. Namikoshi, W. M. Haschek, K. L. Rinehart, W. W. Carmichael, A. M. Dahlem & V. R. Beasley: Structural modifications imparting reduced toxicity in microcystins from Microcystis spp. Toxicon 31, 783-789 (1993)
87. Shima, H., H. Tohda, S. Aonuma, M. Nakayasu, A. A. DePaoli-Roach, T. Sugimura & M. Nagao: Characterization of the PP2Aα gene mutation in okadaic acid-resistant variants of CHO-K1 cells. Proc Natl Acad Sci USA 91, 9267-9271 (1994)
88. Zhang, Z., S. Zhao, F. Long, L. Zhang, G. Bai, H. Shima, M. Nagao & E. Y. C. Lee: A mutant of protein phosphatase-1 that exhibits altered toxin sensitivity. J Biol Chem 269, 16997-17000 (1994)
89. Kaneko, S., H. Shima, T. Amagasa, M. Takagi, T. Sugimura & M. Nagao: Analysis by in vitro mutagenesis of PP2Aα okadaic acid responsive sequences. Biochem Biophys Res Commun 214, 518-523 (1995)
90. Zhang, L., Z. Zhang, F. Long & E. Y. C. Lee: Tyrosine-272 is involved in the inhibition of protein phosphatase-1 by multiple toxins. Biochemistry 35, 1606-1611 (1996)
91. Barford, D.: Molecular mechanisms of the protein serine/threonine phosphatases. Trends Biochem Sci 21, 407-412 (1996)
92. Lohse, D. L., J. M. Denu & J. E. Dixon: Insights derived from the structure of the Ser/Thr phosphatases calcineurin and protein phosphatase 1. Structure 15, 987-990 (1995)
93. Villafranca, J. E., C. R. Kissinger & H. E. Parge: Protein serine/threonine phosphatases. Curr Opin Biotech 7, 397-402 (1996)
94. Kissinger, C. R., H. E. Parge, D. R. Knighton, C. T. Lewis, L. A. Pelletier, A. Tempczyk, V. J. Kalish, K. D. Tucker, R. E. Showalter, E. W. Moomaw, L. N. Gastinel, N. Habuka, X. Chen, F. Maldonado, J. E. Barker, R. Bacquet & J. E. Villafranca: Crystal structures of human calcineurin and the human FKBP12-FK506-calcineurin complex. Nature 378, 641-644 (1995)
95. Bagu, J. B., F. D. Sonnichsen, D. E. Williams, R. J. Andersen, B. D. Sykes & C. F. B. Holmes: Comparison of the solution structures of microcystin-LR and motuporin. Nature Struct Biol 2, 114-116 (1995)
96. Huang, H-B., A. Horiuchi, J. Goldberg, P. Greengard & A. C. Nairn: Site-directed mutagenesis of amino acid residues of protein phosphatase 1 involved in catalysis and inhibitor binding. Proc Natl Acad Sci USA 94, 3530-3535 (1997)
97. Zhang, L. & E. Y. C. Lee: Mutational analysis of substrate recognition by protein phosphatase 1. Biochemistry 36, 8209-8214 (1997)
98. Bagu, J. R., B. D. Sykes, M. M. Craig & C. F. B. Holmes: A molecular basis for different interactions of marine toxins with protein phosphatase-1. J Biol Chem 272, 5087-5097 (1997)
99. Dawson, J. F., H. A. Luu, J. R. Bagu & C. F. B. Holmes: Elucidation of the molecular basis of protein phosphatase-1 sensitivity and protein phosphatase-2B resistance to toxic inhibitors. J Biol Chem Submitted (1999)
100. Quinn, R. J., C. Taylor, M. Suganuma & H. Fujiki: The conserved acid binding domain model of inhibitors of protein phosphatases 1 and 2A: molecular modeling aspects. Bioorg Med Chem Lett 3, 1029-1034 (1993)
101. Gupta, V., A. K. Ogawa, X. Du, K. N. Houk & R. W. Armstrong: A model for binding of structurally diverse natural product inhibitors of protein phosphatases PP1 and PP2A. J Med Chem 40, 3199-3206 (1997)
102. Gauss, C-M., J. E. Sheppeck, A. C. Nairn & R. Chamberlain: A molceular modeling analysis of the binding interactions between the okadaic acid class of natural product inhibitors of the Ser-Thr phosphatases, PP1 and PP2A. Bioorg Med Chem 5, 1751-1773 (1997)
103. Aggen, J. B., J. M. Humphrey, C-M. Gauss, H-B. Huang, A. C. Nairn & R. Chamberlain: The design, synthesis, and biological evaluation of analogues of the Serine-Threonine protein phosphatase 1 and 2A selective inhibitor microcystin LA: rational modifications imparting PP1 selectivity. Bioorg Med Chem 7, 543-564 (1999)

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