Pharmacophore Identification: The Case of the Ser/Thr Protein Phosphatase Inhibitors

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Abstract: This review provides a chronological account of the identification and refinement of the pharmacophore for inhibition of two key serine/threonine protein phosphatases, PP1 and PP2A. The dramatic impact of natural product isolation, molecular modeling, analogue design, biochemical studies, and crystallography on the evolution of the pharmacophore will be described.

Keywords: Protein phosphatase, PP1, PP2A, okadaic acid, microcystin, tautomycin, fostriecin, calyculin.

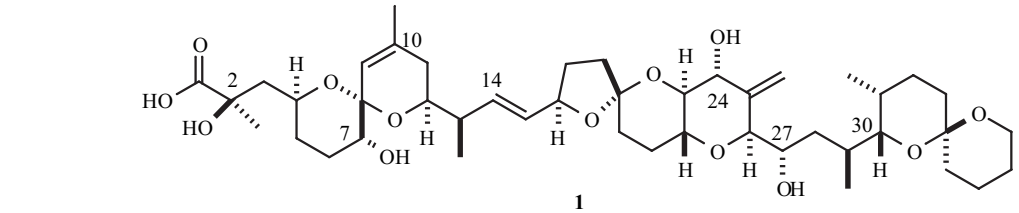
INTRODUCTION

The identification of a pharmacophore can be a key aspect of drug design. A pharmacophore is the general steric and electrostatic interactions that define molecular structures with optimal activity towards a biological target. Medicinal chemists further simplify this concept into the important molecular features, such as hydrogen bond donors, hydrogen bond acceptors, charged groups, hydrophobic groups, and aromatic rings, with their respective 3D geometrical constraints [1]. The process of pharmacophore identification draws on expertise from many different fields, including structural biology, synthetic chemistry, pharmacology, computational biology/chemistry, and others. One particularly illustrative example of this multi-disciplinary convergence is found in a class of toxins that inhibit serine/threonine protein phosphatases.

The protein phosphatases (PPs) are a group of enzymes that act in concert with kinases to regulate a large number of intracellular signaling cascades controlling, for example, cell division, neurotransmission, muscle contraction, and glycogen metabolism. The goal of designing potent, selective inhibitors of serine/threonine PPs has been the subject of a great deal of investigation [2]. This article provides a chronological account of the identification and refinement of the pharmacophore for inhibition of two key serine/threonine protein phosphatases, PP1 and PP2A. The spotlight will be upon the dramatic impact of natural product isolation, analogue design, mutagenesis studies, molecular modeling, and crystallography on the evolution of the PP pharmacophore into its current state.

THE FIRST PP1/PP2A INHIBITORS

In 1988, the natural product okadaic acid (**1**) was reported to be a potent inhibitor of PP1 and PP2A [3], see (Fig. **1**). The discovery immediately revolutionized the ability to study of the role of PPs in cellular process. Okadaic acid was found to be 100-fold selective for PP2A over PP1 with



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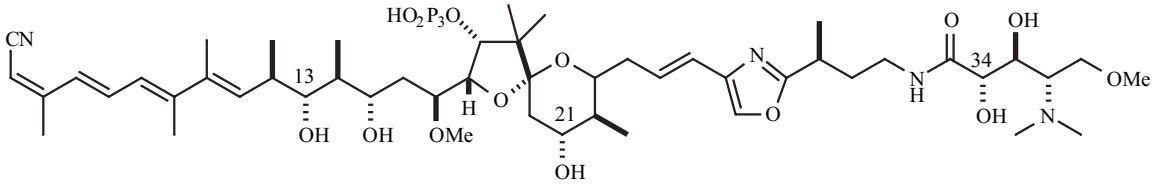

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Fig. (1). Structures of the first two natural product inhibitors of PP1 and PP2A.

IC_{50} = 0.2 nM and IC_{50} = 20 nM, respectively [4]. Soon thereafter, another natural product, calyculin (**2**), was reported to have similar inhibitory activity but no selectivity between the two PPs: PP1 IC_{50} = 2.0 nM and PP2A IC_{50} = 2.0 nM [5]. No structure activity relationship (SAR) for PP1 and PP2A inhibitors was discernable; however, until a third class of inhibitor – a family of cyclic peptides known as the microcystins – made its debut.

The most prominent member of this family, microcystin LR (MCLR) (**3**) (see Fig. **2**), was reported to be a highly

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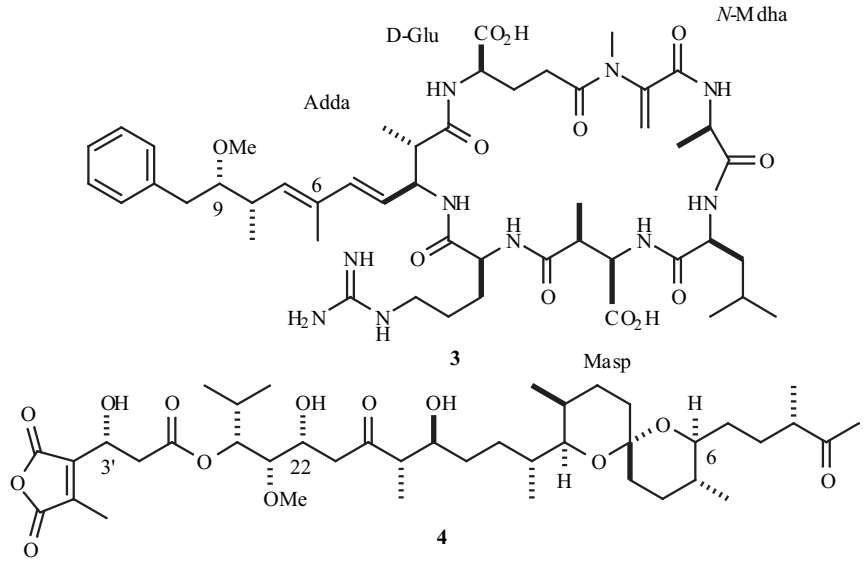

Fig. (2). Structures of MCLR (**3**) and tautomycin (**4**).

potent inhibitor of PP1 and PP2A, with IC_{50} = 0.1 nM for each [6,7]. Comparisons of the biological activity of a variety of naturally occurring microcystin variants gave an interesting first look into SAR for PP1 and PP2A. Isomerization of the C6–C7 olefin of the residue Adda destroyed the activity [8], while replacing the C9 methoxy group with an acetyl ester had no effect [9]. The analogue with a free C9 alcohol was also active; however, esterification of the D-Glu residue resulted in an inactive compound [10]. One more key piece of data became available when the activity of MCLR was compared to that of okadaic acid: they compete for the same binding sites on PP1 and PP2A [7].

By this time, okadaic acid derivatives, both natural and synthetic, began to appear in the literature. An interesting 9,10-epithio okadaic acid was isolated and had inhibitory activity similar to the parent toxin [11]. The C1 methyl ester analogue of okadaic acid was inactive, the two fragments from cleaving okadaic acid at the C14–C15 olefin were only weak inhibitors, and the tetramethyl ether derivative had no activity [12]. Reduction of the C14–C15 olefin of okadaic acid decreased potency 10-fold for PP2A, but removal of the C7 hydroxy group had a minimal effect on activity, while removal of the C2 hydroxyl decreased PP2A potency by 30-fold [13]. At this time, another important PP inhibitor, tautomycin (**4**), was discovered with IC_{50} = 3 nM for PP1 and 30 nM for PP2A, (see Fig. **2**) [14]. This report also provided evidence that tautomycin shared a common binding site with okadaic acid, like MCLR and calyculin. Cantharidin was later shown to be an inhibitor of PP1 and PP2A, although not as nearly potent as the previous four natural products. Further discussions of cantharidin will be omitted, as it has recently been the primary topic of two excellent reviews [2,15].

AN EARLY PHARMACOPHORE FROM MOLECULAR MODELING AND SAR

The first PP pharmacophore was proposed in 1993 and was based on a molecular modeling comparison of MCLR, calyculin, and okadaic acid [16]. Four critical features were proposed: an acidic group, two separate hydrogen bonding sites, and a non-polar side chain. This study was remarkably insightful and rationalized many of the available SAR data. According to the pharmacophore, the corresponding key features on MCLR were the D-Glu carboxylic acid, the D-Glu ring carbonyl, the Adda carbonyl, and the Adda side chain, respectively. Calyculin conformed to the model with the phosphoric acid functionality, oxazole, C34 hydroxyl, and tetraene side chain. The four analogous features on okadaic acid were the carboxylic acid, C4–C8 tetrahydropyran ether, C24 hydroxyl, and C30–C38 spiroketal. Although the correspondence of the suggested acidic groups and non-polar side chains in these inhibitors would prove to be correct based on later structural studies, considerable PP pharmacophore refinements were on the horizon.

More SAR data soon became available, again from the microcystins. MCLR variants from reduction of the *N*-Mdha residue to *N*-Me Ala retained their activity [17]. Three linear peptide analogues devoid of the Adda residue were prepared but were inactive, which added some credence to the necessity of the non-polar side chain feature of the pharmacophore [18]. Another study showed that biological activity was unaffected when varieties of amino acid residues were substituted for the L-Leu and L-Arg residues of MCLR [19]. Indeed, it is the variability of these two residues that is primarily responsible for the diversity of the microcystin family. Interestingly, every discovered member of the microcystin family has been found to be non-selective for PP1 and PP2A inhibition, and at this point, okadaic acid had risen to considerable prominence as a biological tool due to its unique selectivity (greater than 100-fold for PP2A over PP1).

The issue of preferential selectivity for PP1/PP2A inhibition had hardly been addressed when two new inhibitors, fostriecin (**5**) [20], (see Fig. **3**), and thyrsiferyl-23 acetate [21] were reported in 1994. While both of these natural products displayed preferential selectivity for PP2A inhibition, okadaic acid remained the leading choice as a biological probe for PP activity for several reasons. The

selectivity for PP2A by fostriecin was reported to be less than okadaic acid, which slowed the acceptance of fostriecin into the field of phosphatase research. Thyrsiferyl also received less attention because it was much less potent (micromolar range) than the other natural products. No other compounds were able to steal the spotlight from okadaic acid, and later in 1994, the SAR for the C27 hydroxyl of okadaic acid was finally determined: oxidation decreased potency by 230-fold for PP2A and 40-fold for PP1 [22].

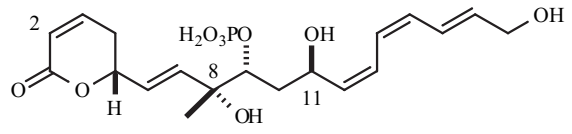

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Fig. (3). Structure of fostriecin (**5**).

SUBSEQUENT INFLUENCE OF BIOLOGY AND X-RAY CRYSTALLOGRAPHY

At that time, a growing body of SAR data began to stimulate interest in refining the pharmacophore. Resistance to toxicity by okadaic acid was observed in variants of CHO cells that contained a C269G mutation in PP2A [23]. A chimeric PP1 mutant, which replaced residues 274–277 (GEFD) with residues 267–270 (YRCG) from PP2A, was screened against okadaic acid, MCLR, calyculin, and tautomycin, and MCLR and calyculin each showed a 4-fold decrease in potency against the chimera, while tautomycin produced no difference [24]. On the other hand, okadaic acid displayed a 10-fold increase in potency. A labeling study confirmed that okadaic acid, MCLR, calyculin, and tautomycin bind PP2A in a mutually exclusive manner, that is they bind competitively at the same site [25]. Although the implications of these three studies were not yet fully understood, they later provided a basis for rationalizing selective inhibition.

In 1995, the X-ray structure of uncomplexed PP1 [26] was disclosed concurrently with the X-ray structure of the MCLR-PP1 complex [27]. According to the X-ray structure of PP1, a β12–β13 loop is situated proximal to the catalytic site (substrate and natural product binding site). This observation, taken with the biological data reported earlier, provided an excellent opportunity to refine the PP pharmacophore to include a new feature on each natural product that contacts this loop. The MCLR-PP1 co-complex was truly a break-through in the field and provided not only invaluable insight into the binding mode of MCLR itself, (see Fig. **4**), but also the other natural products via molecular modeling (see below). The authors described three primary regions on PP1 with which MCLR interacts: a metal binding site (catalytic site), a hydrophobic groove, and the edge of a C-terminal groove, the β12–β13 loop. Indeed, the pharmacophore model could be mapped onto MCLR, and the corresponding residues of PP1 could be distinguished for the first time. These key interactions are depicted in (Fig. **4**) according to the PP1-MCLR complex. Both Arg221 and Tyr272 contact the D-Glu carboxylate (acidic site) of MCLR, Arg96 and Tyr134 are hydrogen bonded to the Masp carboxylate (hydrogen bonding site), Trp206 and Val223 interact with Adda (non-polar side chain), and Cys273 forms a covalent linkage with Mdha (β12–β13 loop-contacting site).

REFINEMENTS TO THE PHARMACOPHORE

In order to produce a more refined pharmacophore model, the next priority became extending the structural data with additional analogues. Two MCLR degradation products were isolated: a linear MCLR from amide cleavage at the Adda and L-Arg connection, and a linear tetrapeptide containing the Adda, D-Glu, Mdha, and D-Ala residues [28]. The linear MCLR displayed a 150-fold decrease in potency, while the tetrapeptide showed only a 20-fold decrease. Indeed, the activity of this tetrapeptide was quite significant and led to further synthetic studies that would probe this result. At the time, it was clear that the tetrapeptide contained nearly all of the elements of the pharmacophore for phosphatase inhibition, which was a contrast to a different series of MCLR analogues, which omitted a key pharmacophore region, the non-polar side chain. Cyclic peptides with L-Cys

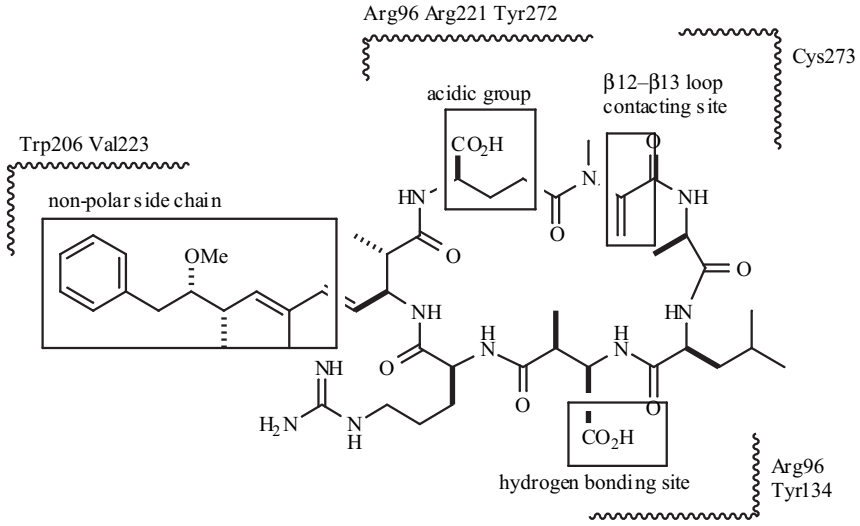

Fig. (4). MCLR with the features of the pharmacophore highlighted and the key PP1 contacting residues depicted according X-ray complex [27].

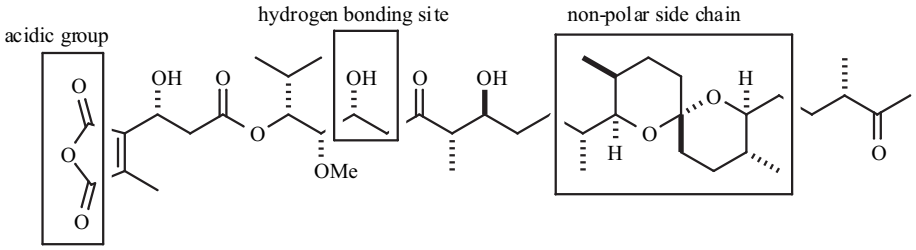

Fig. (5). Mapping the pharmacophore onto tautomycin.

or β-Ala exchanged for Adda produced conformations similar to the parent molecule according to modeling analyses, but showed nearly no activity in PP2A inhibition assays [29,30]. The importance of the non-polar side chain of Adda, in the case of the microcystins, was again evident.

The pharmacophore model was then mapped onto tautomycin for the first time, see Fig. (**5**). Careful molecular modeling overlays of tautomycin with okadaic acid suggested that the anhydride of tautomycin (perhaps in its hydrolyzed form) corresponded to the carboxylic acid of okadaic acid and the acidic group of the pharmacophore. The C6–C14 spiroketal of tautomycin was remarkably similar to the C30–C38 of okadaic acid and possibly served as the non-polar side chain [31]. Additionally, one new SAR of tautomycin was given: the necessity of the C22 hydroxyl for potent phosphatase inhibition.

More comparisons among the natural products were also appearing from mutagenesis analysis. In inhibition assays with a Y272F PP1 mutant, MCLR showed a modest change (< 10-fold increase) in IC_{50}, while calyculin and tautomycin showed 300-400 fold increases in IC_{50} [32]. Also, okadaic acid showed 50-fold decrease in IC_{50}. Although other mutations across the residues 268 to 277 (mainly in the β12–β13 loop) of PP1 were screened, the importance of Tyr272 was profound in the four natural products. In another study, site-directed mutagenesis of the residues in the active site of PP1 (determined from the X-ray data) was performed, followed by inhibition studies [33]. The salient data point that this report added to the growing body of PP knowledge was the critical nature of residues 96 and 221 for inhibition by okadaic acid and calyculin. The importance of these two residues had already been evident in the MCLR-PP1 X-ray structure, but the extension to okadaic acid and calyculin suggested analogous contacts.

EXTENSION OF THE PHARMACOPHORE WITH MOLECULAR MODELING

In 1997, four molecular modeling analyses of the natural products that inhibit PP1 and PP2A were published, and each was based primarily on data available from the PP1-MCLR X-ray structure. Holmes and co-workers suggested a motif for okadaic acid and calyculin binding to PP1 [34]. Okadaic acid was proposed to assume a cyclic shape and mapped onto MCLR with the carboxylate and C30–C38 spiroketal corresponding to the D-Glu carboxylate and Adda, respectively. This model included the C24 alcohol hydrogen bonded to Arg221. Calyculin was also docked into PP1 with a cyclic conformation, but very little discussion of the model was presented.

Armstrong proposed a similar cyclic binding mode for the okadaic acid-PP1 complex [35]. In this model, the okadaic acid C1 carboxylate and the C24 hydroxy group contact Arg96, with the C27 hydroxyl forming a hydrogen bond to Arg221 and the C30–C38 spiroketal occupying the hydrophobic groove. Calyculin also assumed a cyclic conformation, but this arrangement caused the tetraene portion to fit poorly in the hydrophobic groove. Additional contacts were the phosphate and the C35 alcohol with Arg96 and the C34 hydroxy group with Tyr134. The poor fit of calyculin in PP1 was soon reconciled by Koskinen's modeling study, which suggested an alternative extended binding conformation by positioning the calyculin dimethyl amine near Arg221, while maintaining the phosphate-Arg96 contact [36].

A more comprehensive modeling analysis of the PP1/PP2A complexes was reported by Chamberlin and co-workers [37]. An addition to the pharmacophore was suggested: a proximal methyl substituent adjacent to the acidic group. The proposed model for okadaic acid bound to PP1 was similar to Holmes' cyclic model. Calyculin assumed an extended binding conformation in PP1 with the C11 and C13 hydroxyl groups hydrogen-bonded to Arg221. Tautomycin was mapped onto okadaic acid with the anhydride as the acidic group and the C6–C14 spiroketal as the non-polar unit. A key PP1 hydrogen bonding contact was the C22 alcohol of tautomycin with Arg221. Binding conformations for the molecules to PP2A via protein homology modeling provided the first models of inhibitors binding to this phosphatase, for which there are still no experimental structures. No explanation for preferential selectivity for PP2A (or PP1) inhibition was evident from these models.

PHARMACOPHORIC ELEMENTS OF SELECTIVE INHIBITORS

With a growing body of structural data, interest in selective phosphatase inhibitors was now starting to rise, and tautomycin was of considerable interest because of its preferential selectivity for PP1 (>5:1). The hypothesis that tautomycin and okadaic acid map onto one another and adopt similar bound forms was further advanced by two conformational analyses [38,39] and studies of a synthetic tautomycin analogue bearing the C30–C38 spiroketal of okadaic acid [40]. Twenty-two tautomycin analogues offered additional SAR data [41]. Specifically, the essential nature of the C22 hydroxyl (hydrogen bonding site in the pharmacophore) and the C1'–C7' unit (acidic group) of tautomycin were highlighted. One of the twenty-two tautomycin analogues was a highly truncated derivative that

displayed phosphatase inhibition with IC_{50} = 40 μM for PP1 and PP2A. The potency of this truncated analogue was surprising, since the compound lacks both the tautomycin spiroketal (non-polar side chain) and the pharmacophoric hydrogen bonding feature as in Fig. (**5**).

Despite earlier reports of modest selectivity, fostriecin was found to be a highly selective inhibitor of PP2A: IC_{50} = 3.2 nM for PP2A and 131 μM for PP1 [42]. Okadaic acid was thusly dethroned as the most selective PP2A inhibitor (100-fold versus >10,000-fold). At the same time, combinatorial library synthesis was conducted using the pharmacophore model to guide scaffold design [43]. This unique report was the first example of designed phosphatase inhibitors, and more refined applications to analogue creation soon followed. In 1999, a series of microcystin LA (MCLA) variants that were designed to preferentially favor selectivity for PP1 were disclosed [44]. Seven-to-one selectivity for PP1 was achieved in a designed synthetic MCLA analogue with a cyclohexyl-Ala in place of L-Leu; this result was explained by differential contacts to the residues of the β12–β13 loop of PP1 and PP2A as shown in (Fig. **6**).

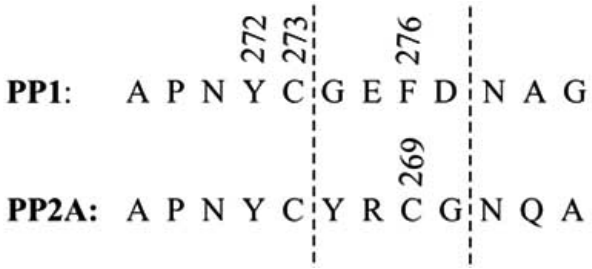

Fig. (6). Sequences of the β12–β13 loop in PP1 and PP2A. Key residues are numbered. The residues enclosed by dashed lines have been suggested to be responsible for the preferential selectivity of some of the natural products.

Contrary to this modest success, biological investigations offered conflicting evidence for the role of the β12–β13 loop as the sole determinant of selectivity for PP1 over PP2A, see (Fig. **6**). A chimeric PP1 mutant containing the β12–β13 loop of PP2A showed no difference in IC_{50} values compared to wild-type PP1 when screened against MCLR, tautomycin, calyculin, and fostriecin [45]. This lack of differential potencies was also a divergence from an earlier study with a different chimeric PP1 mutant containing only four residues of the β12–β13 loop exchanged for PP2A [24]. Thus, the exact features of the pharmacophore model that control selectivity for PP1 versus PP2A were not yet apparent.

MORE DESIGNER ANALOGUES AND STRUCTURAL DATA

Additional designer analogues soon appeared and directly tested the role of specific features of the pharmacophore model. Biotinylated okadaic acids were rationally designed based on the existing SAR data; the C7 hydroxyl of okadaic acid was the ideal biotin linkage site compared with the C1 carboxylate, C24 or C27 hydroxy groups [46]. Okadaic acid and tautomycin derivatives with only the hydrophobic spiroketal group (non-polar side chain feature of the pharmacophore) showed no inhibition of either PP1 or PP2A [47]. Motuporin, a cyclic pentapeptide relative of the microcystins, and two analogues containing an L-Ala instead of the Mdha residue (β12–β13 loop-contacting feature of the pharmacophore) were synthesized and displayed potent inhibition for PP1 [48]. Two tautomycin derivatives, one lacking the C1–C5 segment and one with the C28–C38 unit of okadaic acid in place of the tautomycin spiroketal, were prepared; and each showed a major loss in potency of inhibition for PP1 and a minor loss for PP2A [49].

When the co-crystal structure of okadaic and PP1 was published in 2001, (see Fig. **7**), the common binding motif for the natural products to PP1 was apparent [50]. The C30–C38 spiroketal of okadaic acid occupies the hydrophobic groove, making two contacts with Ile130 and Trp206. With the okadaic acid backbone in a cyclic conformation, the terminal α-hydroxy carboxylic acid is within the metal binding site, and similar to the MCLR-PP1 structure, interacts with Arg96 and Tyr272. Hydrogen bonding between the C24 hydroxyl of okadaic acid and Arg221 is also observed. Other contacts are the Tyr134 with the C25 *exo*-cyclic olefin and both Cys273 and Phe276 with the C10 methyl group of okadaic acid. One contrast with the X-ray complex of MCLR to PP1 was the lack of a contact to Arg96 on the okadaic acid backbone that corresponded to the acid of the Masp residue of MCLR. This difference was offered as an explanation for the weaker binding of okadaic acid to PP1. The authors also postulated that an additional reason for the inherent selectivity of okadaic acid was the interactions with the β12–β13 loop at Tyr272, Cys273, and Phe276.

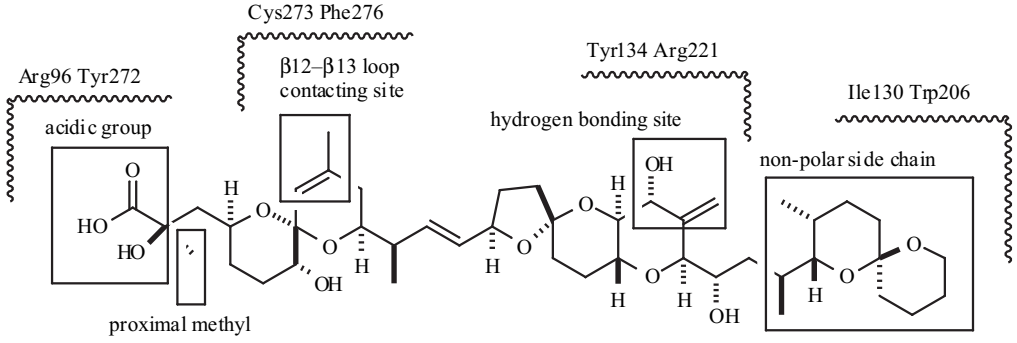

Fig. (7). Okadaic acid with the features of the pharmacophore highlighted and the key PP1 contacting residues depicted according X-ray complex [50].

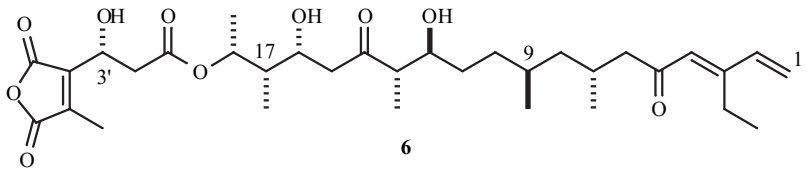

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Fig. (8). Structure of tautomycetin (**6**).

The importance of the contacts with the β12–β13 loop was further emphasized when fostriecin was screened against a C269F PP2A mutant (Phe is the corresponding PP1 residue): a four fold decrease in IC_{50} value was measured [51]. A C16–C38 okadaic acid fragment, which lacked the β12–β13 loop and the metal binding site contacting regions, was completely inactive, while a C1–C27 fragment, which lacked only the hydrophobic region, had a 50-fold increase in IC_{50} against PP2A and an 800-fold increase for PP1 when compared to okadaic acid [52]. Two MCLR metabolites from conjugation at the Mdha residue (β12–β13 loop-contacting feature of the pharmacophore) with cysteine and glutathione were approximately as active as the parent compound [53]. Finally, a new benchmark for preferential selectivity for PP1 was discovered in the natural product tautomycetin (**6**), with IC_{50} = 1.6 nM for PP1 and 62 nM for PP2A [54]. Tautomycetin is clearly a structural cousin of tautomycin with the primary difference in the presence of a diene tail instead of the spiroketal of tautomycin, (see Fig. **8**).

The crystal structure of the calyculin-PP1 complex was then published, which resolved the aforementioned issue about the binding conformation of calyculin [55]. Indeed, calyculin adopts an extended binding conformation, rather than a more cyclic form, and orients the tetraene in the hydrophobic groove with a contact to Val223, (see Fig. **9**). The phosphate is situated in the metal binding site and contacts Arg96, Arg221, and Tyr272. Both the C13 and C15 alcohols form a hydrogen bond to Arg221, and the opposing C12 and C14 methyl groups contact Tyr134. The authors compared the conformation of the β12–β13 loop to the okadaic acid-PP1 and the MCLR-PP1 X-ray structures and found a near prefect match between the loops in the PP1 co-complexes with calyculin and okadaic acid. The discrepancy in the MCLR-PP1 structure is from the movement of Cys273 in order to form a covalent linkage at the Mdha residue of MCLR. No hypothesis for the selectivity for PP1/PP2A was put forth in this comparison.

NEW APPLICATIONS OF THE PHARMACOPHORE

Interest in the natural products displaying greater selectivity for PP inhibition continued unabated in 2002. One careful analysis suggested that the preference for selectivity towards PP2A by okadaic acid arises from favorable contacts between the C9–C10 vinyl methyl group and the β12–β13 loop of PP2A [56]. As for preferential selectivity for PP1, a conformational comparison between the C1–C17 fragment of tautomycin and the C1–C11 fragment of tautomycetin suggests that the two are essentially superimposable [57]. A pharmacophore model for fostriecin was proposed in which the triene moiety served as the hydrophobic group in the pharmacophore, and the phosphate was the acidic group, (see Fig. **10**) [58]. Also, the C11 hydroxyl was suggested by docking experiments to form a hydrogen bond to Arg221, and because C11 acetylation inactivates the fostriecin derivative. The unsaturated lactone of fostriecin was determined to reside in a site similar to the MCLR Mdha residue. Finally, the C8 methyl of fostriecin was hypothesized to be the proximal methyl group feature of the pharmacophore (similar to the C2 methyl of okadaic acid) and was rationalized for the preferential selectivity for PP2A.

Support for the idea that the non-polar side chain and the acidic group are the most critical elements of the pharmacophore surfaced when it was found that microcystin analogues containing only these elements (side chain of Adda and D-Glu carboxylate) were reasonably potent (low micromolar) inhibitors of PP1 and PP2A [59]. Second generation truncated microcystin analogues containing additional functionality, but not additional features of the pharmacophore, also displayed comparable inhibition with a trend for PP2A selectivity [60]. On the other hand, further simplification of the analogues by alterations to Adda resulted in inactive derivatives. From these two studies, one of the most potent truncated microcystins was Adda D-Ala (**7**), (see Fig. **11**) [61]. The conclusion was that only two

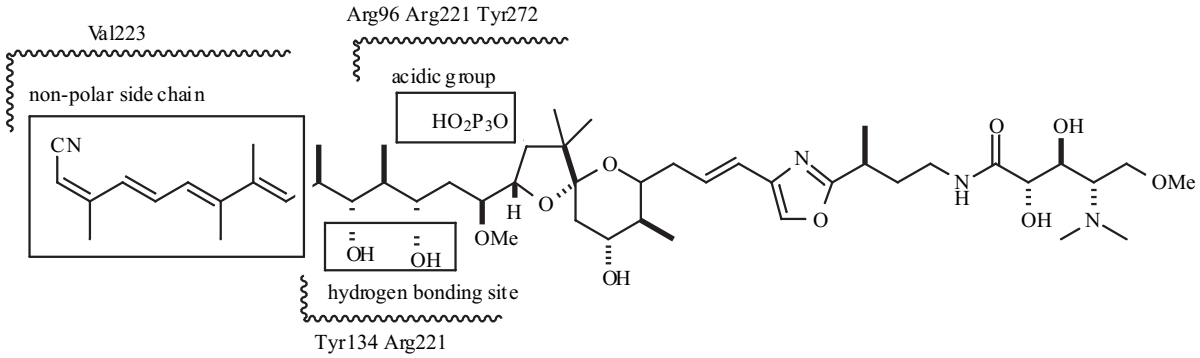

Fig. (9). Calyculin with the features of the pharmacophore highlighted and the key PP1 contacting residues depicted according X-ray complex [55].

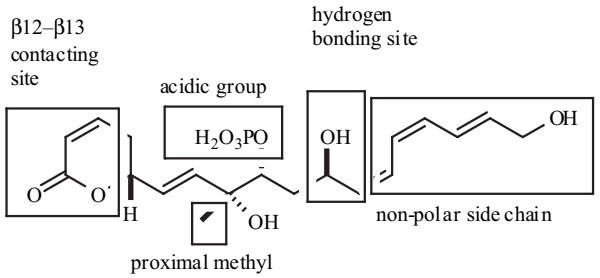

Fig. (10). Mapping the pharmacophore onto fostriecin.

features of the pharmacophore – the non-polar side chain and the acidic group – are alone sufficient for reasonably potent inhibition.

The biological activity of a series natural and synthetic calyculin derivatives provided SAR for many of the important interactions in the calyculin-PP1 crystal structure and indirectly added weight to the "two feature" idea [62]. One key conclusion about calyculin was drawn: the phosphate group (acidic group), the C13 hydroxy group (hydrogen bonding site), and the tetraene (hydrophobic group) were essential for PP1 and PP2A inhibition. This hypothesis was supported by the observed activity of a highly truncated calyculin derivative, hemicalyculin (**8**), with inhibitory potency nearly equal to the parent compound, (see Fig. **11**). Another highly potent truncated derivative (**9**) emerged from molecular modeling studies on tautomycin [63]. This report also challenged the idea of a binding motif that is similar for tautomycin to okadaic acid and instead found more similarities with calyculin. The derivative (**9**) was designed to retain the proposed acidic group and hydrophobic region of tautomycin, (see Fig. **11**).

MOST RECENT SAR AND ANALOGUES

Additional analogues were prepared to investigate the importance of the features of the pharmacophore. A group of fostriecin analogues arose from PP2A homology modeling [64]. Removal of the phosphate group of fostriecin resulted in an inactive derivative, acetylation of the C11 alcohol decreased potency by 50-fold, and removal of the unsaturated lactone decreased potency by 200-fold. Clearly the acidic group, the hydrogen bonding group, and the β12–β13 loop-contacting region of fostriecin were important for potent inhibition. In order to further investigate the role of the acidic group and the proximal methyl group (see below), full-sized synthetic variants of tautomycin with modifications only in the C1'-C7' region were prepared [65]. No changes in selectivity were observed, even when the C1–C3 end of okadaic acid was substituted on tautomycin; the data suggest that the specific identity of the acidic group and the proximal methyl group in pharmacophore do not play a significant role in selectivity.

The influence of the β12–β13 loop in selective inhibitor binding was also further probed by the preparation of new PP1 mutants. Systematic mutation of each residue in the β12–β13 loop showed that changing Cys273 caused the largest change in PP1 sensitivity for okadaic acid and MCLR [66]. Also, the conformation of the β12–β13 loop in the previously reported MCLR-PP1 X-ray structure was shown to be atypical due to the covalent linkage formed between the inhibitor and PP1. As for PP2A, sequential mutation of the cysteine residues to serine lead to the identification of Cys269 in the β12–β13 loop as the target for a fostriecin congener [67]. Clearly, the importance of the β12-β13 loop was apparent, but a unifying explanation for preferential selectivity for PP1 and PP2A was not evident.

SUMMARY: THE CURRENT PHARMACOPHORE

The pharmacophore for phosphatase inhibition has undergone considerable revision since it first arose in 1993. There have been a total of six elements proposed: an acidic group, a proximal methyl group, two hydrogen bonding sites, β12–β13 loop-contacting region, and a non-polar side

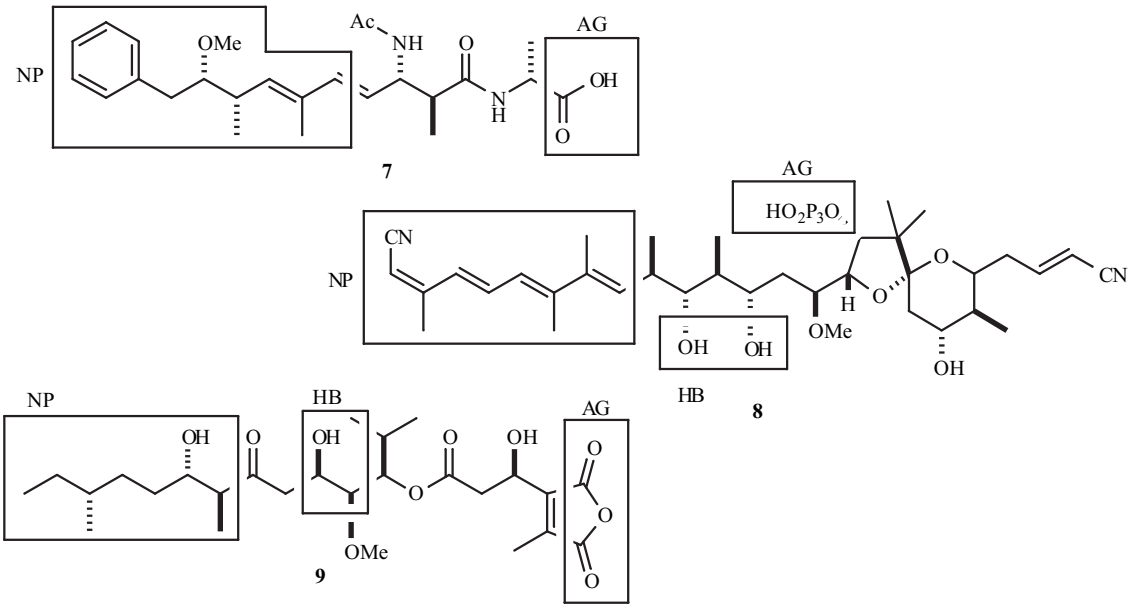

Fig. (11). Structures of three truncated PP1 and PP2A inhibitors. The parts of the pharmacophore are labelled: AG = acidic group, HB = hydrogen bonding site, and NP = non-polar side chain.

chain (or hydrophobic segment). The key elements of the pharmacophore for potent phosphatase inhibition are the acidic group, the β12–β13 loop-contacting region, a hydrogen bonding site, and the hydrophobic group, (see Fig. **12**). They are clearly present in all of the natural products. Perhaps the most critical features of the pharmacophore are the acidic group and the hydrophobic group, as the two elements were designed into a group of potent, yet simplified natural product derivatives.

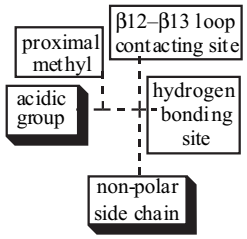

Fig. (12). The current pharmacophore. The most important elements are highlighted in bold.

The elements of the pharmacophore that are responsible for selective phosphatase inhibition are still only poorly defined, and two working hypotheses for preferential selectivity for PP2A have arisen. The proximal methyl group of the pharmacophore can be mapped onto okadaic acid and fostriecin, and the feature has been continually suggested to control selectivity. This rationale is not consistent with all the SAR, because the presence of the group on synthetic tautomycin analogues does not impart any selectivity. The other explanation for selectivity for PP2A relies heavily on the contacts with the β12–β13 loop. While the role of the β12–β13 loop in selectivity has been demonstrated from mutagenesis studies and SAR, the specific structural elements of the inhibitors that control PP1/PP2A selectivity remain unclear.

The issue of PP1 selectivity has not yet been addressed due to a lack of highly selective PP1 inhibitors. Indeed, some selectivity for PP1 has been engineered into microcystin analogues by modulating the β12–β13 loop contacts. A simple comparison of the structure of tautomycetin, the most selective PP1 inhibitor, to tautomycin would suggest that the non-polar side chain or the β12-β13 loop-contacting region of the pharmacophore is responsible for selectivity. One possible rationale for the non-polar side chain of tautomycetin controlling selectivity for PP1 is that the C1–C5 dienone of tautomycetin serves has an electrophilic site for a cysteine residue (Cys127) that is present in the hydrophobic groove of PP1 but not PP2A (the equivalent residue is serine). Reversible conjugate addition in the tautomycetin-PP1 complex could thus slow the off-rate and lower the K_i for PP1 inhibition.

The newest, structurally distinct phosphatase inhibitor, spirastrellolide A (**10**) in (Fig. **13**), has appeared in the literature with IC_{50} = 50 nM for PP1 and IC_{50} = 1 nM for PP2A [68]. Attempts to reconcile the structure and 50-fold selectivity for PP2A with the pharmacophore have yet to be made, although the C47 carboxylate is almost certainly the acidic group of the pharmacophore. Either the C3–C7 tetrahydropyran, C13–C21 spiroketal, or C27–C35 trioxadispiroketal may be a surrogate for the other known non-polar side chains. Four alcohols are displayed along the backbone of spirastrellolide and may serve as the hydrogen bonding site of the pharmacophore. Future SAR data on this new natural product and additional studies on the existing ones will move the pharmacophore beyond its current limitations, particularly with regard to its major deficiency – predicting PP1/PP2A selectivity.

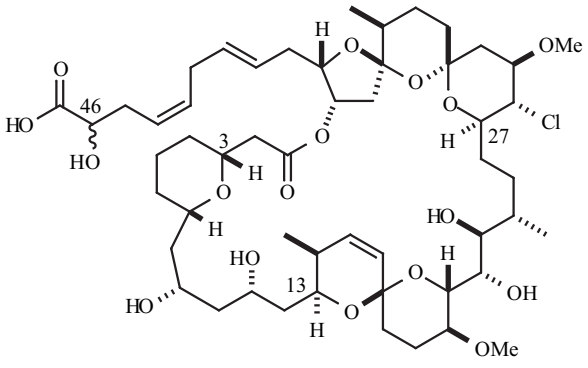

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Fig. (13). Structure of spirastrellolide A (**10**).

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ABBREVIATIONS

PP	=	Protein phosphatase
PP1	=	Protein phosphatase 1
PP2A	=	Protein phosphatase 2A
SAR	=	Structure activity relationship
MCLR	=	Microcystin LR
MCLA	=	Microcystin LA

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