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Perspective

Serine−Threonine Protein Phosphatase Inhibitors: Development of Potential Therapeutic Strategies

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1. Introduction

The regulation of cellular processes by the modulation of protein phosphorylation/dephosphorylation is fundamental to a large number of, if not all, physiological functions. The phosphorylation level of a given protein is governed by the balance between two enzyme activities: protein kinases which transfer phosphate from ATP to the protein (phosphorylation) and protein phosphatases which catalyze the reverse reaction (dephosphorylation). It is considered that over 30% of all cellular proteins are subject to phosphorylation at one or more residues, and eukaryotic cells have been estimated to possess 575 kinase genes or 2% of the genome (given that the estimated total number of genes is 30−40000).[1] Protein kinases are integral components of cell signal transduction pathways, and the regulation of many of these protein kinases in response to extracellular signals is well understood.

Virtually every disease has, at its core, a deficiency in cellular signaling such that protein kinases are considered viable targets for the design of novel therapeutics. Understanding the role of protein phosphatases in these systems has lagged considerably behind that of protein kinases. Indeed, until recently, protein phosphatases were considered relatively nonspecific, unsophisticated enzymes that existed only to reverse the actions of protein kinases. It is now widely acknowledged that the regulation of protein phosphorylation requires the coordinated control of both kinases and phosphatases and that the regulation of phosphatases is as complex and elegant as that of kinases, perhaps more so. Genomic data suggests that there are 13 genes and 15 proteins in the PPP family and an additional 10 genes in the PPM family. Thus the serine/threonine protein phosphatases comprise <0.1% of the human genome.[2]

To view protein phosphatases as simply molecular "off switches" is clearly an oversimplification of their roles as "rheostats" governing the fine control of some processes, and in some cases the major point of control is emerging. Consequently, protein phosphatases are beginning to be linked with the etiology or control of disease processes. In the past decade there has been a substantial increase in effort to elucidate their biological roles, and thus their potential medical implications. Protein phosphatases that dephosphorylate tyrosine or serine and threonine represent distinct classes of enzyme, and each class warrants separate consideration. In this review, discussion is limited to only the serine/threonine protein phosphatases. We discuss some of the opportunities, implications, and limitations of utilizing the "*inhibition of serine−threonine protein phosphatases as a therapeutic strategy*".

2. Classification of Protein Phosphatases

Serine/threonine protein phosphatases can be classified into two families according to homology between

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Table 1. PPP and PPM Families of Serine/Threonine Phosphatases

protein phosphatase major catalytic subunits	characteristic	isoforms	regulatory proteins	refs
PP1	IC_{50} for okadaic acid is 10−50 nM	α, β (or δ), $\gamma1$, $\gamma2$	I-1, DARPP-32, I-2, G_m, G_l, M_{110}+M_{21}, NIPP-1, RIPP-1, R110, Spinophilin, Yotiao, PNUTS, PTG, AKAP220, sds22, p53BP2, GAC1, splicing factor, R5, CPI17	4−16
PP2A	IC_{50} for okadaic acid is 0.5 nM	α, β	A subunit, B subunit (PR55, B56, PR72 families) SV40 small T antigen, Polyoma middle T antigen, Polyoma small T antigen, SET, eRF1, NCp7, Vpr, cyclin G, Tau, p107, CamPKIV, PKC, p70 S6 kinase, Phap-1, HRX, APC protein, axin, alpha4, PTPA, G-substrate	17−35
PP2B	Ca(II)-dependent; IC_{50} for okadaic acid is >2000 nM	α, β, γ	B subunit, Calmodulin, AKAP79/150, FKBP12, cyclophilin, Cain.	36−39
PP2C	Mg(II)-dependent; not inhibited by okadaic acid			
PP3	IC_{50} for okadaic acid is 4 nM			

novel catalytic subunits	homology	refs
PP5	PP1, PP2A, PP2B	3
RdgC/PPEF	PP5	3
PP7	PP5	3
PPY, PPZ, PPQ	PP1	3
PP4	PP2A	3
PPG	PP2A	3
PP6, PPV, SIT4	PP2A	3

Table 2. Novel Members of the PPP and PPM Families of Serine/Threonine Phosphatases

protein phosphatase	homology	regulatory proteins[a]	refs
PP5	PP1, PP2A, PP2B	-	3
RdgC/PPEF	PP5	-	3
PP7	PP5	-	3
PPY, PPZ, PPQ	PP1	-	3
PP4	PP2A	-	3
PPG	PP2A	-	3
PP6, PPV, SIT4	PP2A	-	3

[a] Hyphen (-) = none yet reported.

the amino acid sequences of the catalytic subunits. Within each gene family the major catalytic subunits represent the most highly conserved enzymes known, yet they have considerable functional diversity.[3] While these catalytic subunits have a number of isoforms and in some cases distinct subcellular locations, as listed in Table 1, they are all highly homologous, and their existence alone cannot account for the diversity and specificity of phosphatase action. Moreover, this diversity of function and specificity of action is derived essentially from the association of distinct and diverse regulatory proteins with the catalytic subunits. The major representatives of the so-called PhosphoProtein-Phosphatase (PPP) family (Table 1) comprise PP1, PP2A, and PP2B, while the principle member of the PPM family is PP2C. The latter family is characterized by an absolute requirement for a metal ion, particularly magnesium, for activity. While these protein phosphatases constitute the majority of serine/threonine dephosphorylation in all eukaryotic cells, a number of additional novel members of the PPP family have been discovered (largely by molecular cloning), which are expressed at relatively low abundance[3] and are minor contributors to overall dephosphorylation.[3] Nonetheless, these more obscure phosphatases (Table 2) may still lend themselves as good targets for drug design.

Although these phosphatases share a common catalytic domain structure, PP1 and PP2A can be distinguished from PP2B (Calcineurin) in that the latter enzyme is dependent upon Calmodulin/Ca^{2+} for maximal activity.[36] Similarly, the effects of PP1 and PP2A can be distinguished by two heat stable proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2), which bind to the catalytic subunit and uniquely inhibit PP1.[40] A number of other distinct proteins (regulatory subunits) (see Table 1) also bind the catalytic subunit of PP1 and serve to direct the enzyme toward distinct subcellular locations and/or modify the activity toward specific substrates.[32] Despite the lack of primary structure homology between these regulatory proteins, a PP1 binding motif (RVXF) has been found in the majority of these, including inhibitor-1, suggesting that the same domain is required for regulatory binding.[41] PP2A exists in vivo as a trimeric complex. The highly conserved A regulatory subunit serves the role of a core scaffold protein with heterogeneity being introduced by the association of other proteins with the AC core. A number of B regulatory subunits have been identified which have little primary sequence homology, but as with PP1 regulatory subunits, these are believed to be involved in the subcellular targeting of PP2A and the modulation of substrate dephosphorylation.[32] PP2B consists of two tightly associated subunits, calcineurin A (60 kDa) and calcineurin B (19 kDa). The A-subunit shares high sequence homology with PP1 and PP2A, while the B-subunit contains four Ca^{2+} binding sites.[42]

Members of the PPM family are also widely distributed but share no primary sequence homology with the PPP family.[3] PP2C is the predominant member of this family and is present in all mammalian cells. Other members identified to date are pyruvate dehydrogenase phosphatase, ABI1, KAPP-1, and SPOIIE phosphatase. Activity of PP2C is dependent upon Mg^{2+} with half-maximal activation being produced by physiological levels of Mg^{2+}. Thus PP2C is considered to be constitutively active in vivo. It also appears to be monomeric, and no regulatory proteins or mechanisms have thus far been identified. Compared with the PPP phos-

Table 3. Endogenous Protein Inhibitors of the Catalytic Subunits of PP1 and PP2A

inhibitor (source)	PP1	PP2A	ref[a]
DARPP-32 (human brain, kidney)	0.45 nM	NI[b]	48
inhibitor-1 (mammalian tissues)	0.45 nM	NI	48
inhibitor-2 (mammalian tissues)	0.8 nM	NI	48
NIPP-1 (eukaryotic nuclei)	<1 pM	NI	48
I-1^{PP2A} (mammalian nuclei)	NI	4 nM	48
I-2^{PP2A} (mammalian nuclei)	NI	2 nM	48

[a] And references therein. [b] NI = no inhibition.

phatases, substrates for PP2C appear to be restricted and overlap with the PPP family such that the specific roles for PP2C remain unclear.[3] However, a common theme of all the PPM phosphatases is their involvement in the molecular response to cellular stress.[43]

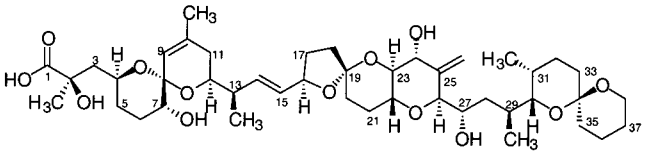
3. Natural Phosphatase Inhibitors

While many protein phosphatase inhibitors are known, only a few show potential for therapeutic use (see section 5). However, by understanding the interaction and mechanism involved in the inhibition of this family of enzymes, new agents that are better designed for therapeutic use may evolve. To achieve this goal we need to understand the role of protein phosphatases in biological reactions, the mechanism of inhibition, and methods of modifying old inhibitors or synthesizing new ones.

Endogenous Inhibitors. A number of intracellular endogenous inhibitors of the serine−threonine protein phosphatases have provided insight into the regulatory mechanisms governing phosphatase activity (Table 3).[44−47] For example, PP1 is regulated by the heat stable proteins, I-1, the related homologue DARPP-32 (dopamine and cAMP regulated phosphoprotein of M_r 32, 000), and I-2. Phosphorylation of I-1 (at Thr-35) or DARPP-32 (at Thr-34) by cAMP-dependent protein kinase (PKA) converts them into potent inhibitors of PP1. PP2A on the other hand is regulated via its own specific inhibitor-1 (I-1^{PP2A}) and inhibitor-2 (I-2^{PP2A}) proteins. No endogenous inhibitors of PP2B or PP2C are known.

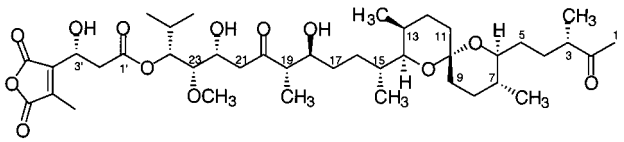
Exogenous Inhibitors. Numerous exogenous inhibitors of protein phosphatases are also known (Table 4), most of which are natural toxins. These toxins are often utilized and/or secreted as a predatory defense mechanism. Interestingly, most of these agents appear to target PP1 and PP2A, but not PP2B. In the case of PP1 and PP2A, the major inhibitors and their synthetic analogues are grouped together and called the okadaic acid class of compounds. As shown in Table 4, the origin, potency, and selectivity of these inhibitors is as diverse as their individual structures.

Okadaic acid (**1**) is a marine polypeptide and was the first of these inhibitors discovered.[55,56] It was initially isolated from the marine sponges *Halicondria okadai* and *Halicondria melanodocia* but is produced by dinoflagellates and is the causative agent of diarrhetic seafood poisoning.



1

Tautomycin (**2**) was isolated from *Streptomyces spiroverticillatus* and exhibits antifungal activity and hepatotoxicity and is the first small molecule inhibitor to display preferential inhibition of PP1 (5-fold versus PP2A inhibition).[58] Tautomycetin (**3**) is a close analogue, but no inhibition data has yet been reported.[59]



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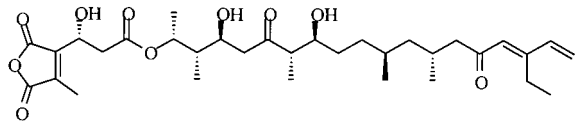


3

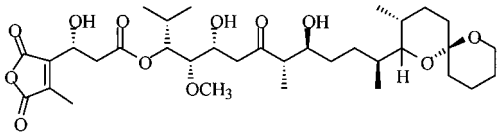
The synthesis of an intriguing okadaic acid-tautomycin hybrid, okadamycin (**4**), has recently been reported.[57]

Table 4. Protein Phosphatase Inhibition by Natural Products

	IC$_{50}$						
inhibitor (source)	PP1	PP2A	PP2B	PP2C	PP4	PP5	ref
okadaic acid (**1**) (dinoflagellates)	3 nM	0.2−1 nM	>10 mM	NI	0.2 nM	-	48
tautomycin (**2**) (bacteria)	0.7 nM	0.7 nM	∼70 mM	NI	-	-	48
microcystin-LR (**5**) (blue-green algae)	0.1 nM	0.1 nM	-	NI	8 pM	>1 nM	48
nodularin-V (**6**) (blue-green algae)	1.8 nM	0.03 nM	8.7 mM	NI	-	-	48
calyculin (**7**) (marine sponge)	0.3−0.7 nM	0.2−1 nM	>10 mM	NI	-	<3 nM	48
thyrisiferyl-23 acetate (**8**) (red algae)	>1 mM	4−16 μM	>10 mM	-	-	-	48
fostriecin (**9**) (bacteria)	131 μM	3.4 nM	-	-	-	-	48
cantharidin (**10**) (blister beetles)	0.5−2 μM	0.2 μM	>1 mM	-	-	-	48
cantharidic acid (**11**)	0.6 μM	0.05 μM	-	>1 M	-	-	48
palasonin (seeds) (**12**)	0.7 μM	0.12 μM	-	>1 M	-	-	49
isopalinurin (**13**) (marine sponge)	*a*	*a*	-	-	-	-	50
dragmacidins (**14**, **15**) (marine sponge)	*a*	*a*	-	-	-	-	51
discorhabdin P (**17**) (marine sponge)	-	-	1.15 μM	-	-	-	52
FK506 (**18**) (bacteria)			0.5 nM				53
cyclosporin-A (**19**) (fungus)			5 nM				53
cyclolineopeptide-A (**20**) (CLA) (linseed oil)			5−10 μM[b]				54

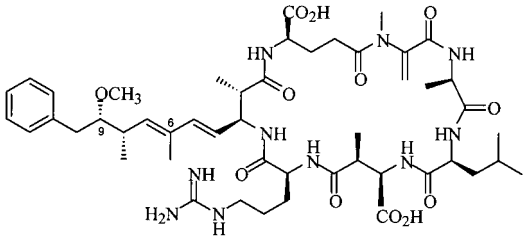
[a] Potency is described as "high micromolar to millimolar". [b] CLA at concentrations of 5−10 mM was able to inhibit the calcineurin activity of activated lymphocyte extracts (see ref 52); NI = no inhibition, hyphen (−) = not determined.

Although it is claimed to be a potent inhibitor of PP2A, no substantiating data was reported.



4

The cyclic peptides, exemplified by the microcystins (microcystin-LR, (**5**)) and the nodularins, were initially isolated from blue-green algae and are essentially equipotent inhibitors of PP1 and PP2A, but poor inhibitors of PP2B and PP2C.[60−62] Motuporin, also known as nodularin-V (**6**), was isolated from a marine sponge.[63] The microcystins have the general structure cyclo(D-Ala-L-**X**-D-*erythro*-β-methylisoasp-L-**Y**-Adda-D-isoGlu-*N*-methyldehydroAla) where **X** and **Y** are variable L-amino acids. Nodularin lacks the variable L-amino acids and D-Ala while the *N*-methyldehydroAla is replaced by the methyl analogue, *N*-methyldehydroaminobutyric acid.



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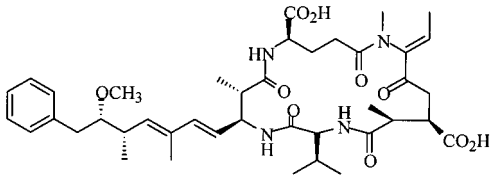
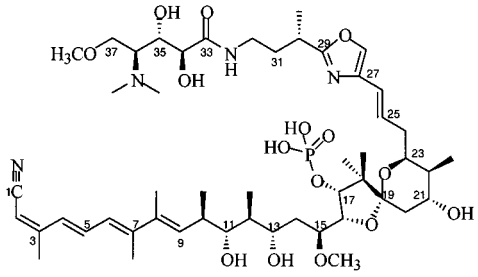


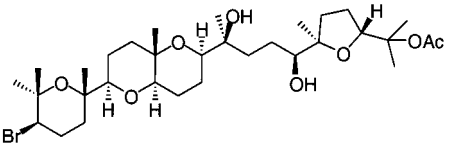
6

The calyculins A to H (calyculin-A (**7**)) were isolated from the marine sponge *Discodermia calyx*[64−67] differing only in the C2 and C6 double bond geometries. Calyculins A−H are equipotent inhibitors of PP1 and PP2A.



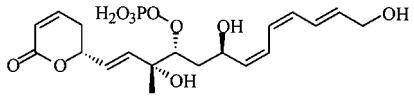
7

Thyrisiferyl-23-acetate (**8**) was isolated from the red algae, *L. obtusa*, and is a selective PP2A inhibitor. No structure−activity studies have been reported for this compound.[68]



8

Fostriecin (**9**) is a structurally novel phosphorus containing antibiotic that was discovered in a fermentation broth of a previously undescribed subspecies of *Streptomyces pulveraceus* (subspecies *fostreus*).[69] It is the most selective small molecule inhibitor of the serine/threonine phosphatases yet discovered. Fostriecin displays 40000-fold selectivity for PP2A over PP1.



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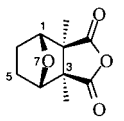
Cantharidin (**10**) is found in over 1500 species of blister beetles including *Lytta vesicatoria* (L.) which occurs around the Mediterranean area, *Lytta tenuicollis* (Pallas) in India, *Mylabris* spp. in India and China, and *Epicauta* spp. in Asia and North America. Cantharidin and *Lytta vesicatoria* share the same common name of Spanish Fly. Cantharidin was found to bind strongly to the cantharidin binding protein, which was subsequently shown to be PP2A. Cantharidin is a moderate inhibitor of PP1 and PP2A and a weak inhibitor of PP2C. Similar inhibitory values have been reported for the closely related cantharidic acid (**11**) and palasonin (**12**).[70]



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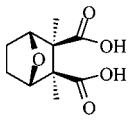

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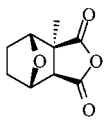
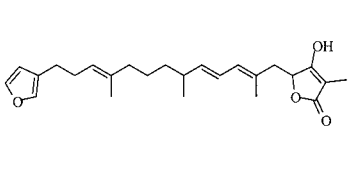


12

There are other small molecule inhibitors of PP1 and PP2A that are structurally simpler than the main okadaic acid class of compounds including isopalinurin (**13**)[50] and dragmacidin-D and -E (**14**, **15**).[51] Although these compounds are described as phosphatase inhibitors, with dragmacidin-D (**14**) portrayed as a selective inhibitor of PP1, the potency of these agents is quite low (high micro- to millimolar range), with no actual values reported. The poor stability of these compounds under assay conditions may also confound such data.



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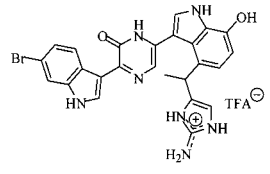

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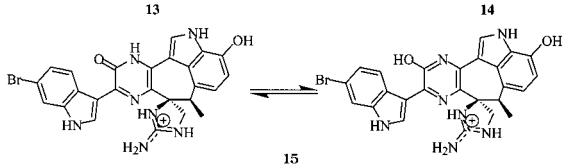

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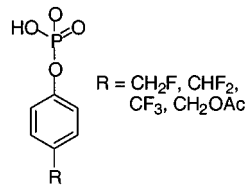
The phenyl phosphates (**16**) represent the first reported (mechanism based) small molecule inhibitors of PP2B, although only weak inhibitors with a K_i of 44 mM when R = CH_2F.[52] Discorhabdin P (**17**) was isolated

from a deep water Caribbean sponge, genus *Batazella*, and was found to be a 1.15 μM inhibitor of PP2B.[71]



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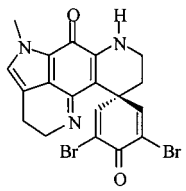

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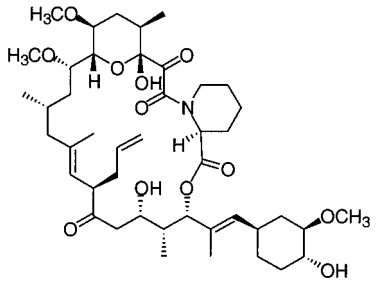
FK506 (**18**) was isolated from the bacteria *Streptomyces* and was found to be a 0.5 nM inhibitor of PP2B.[53]



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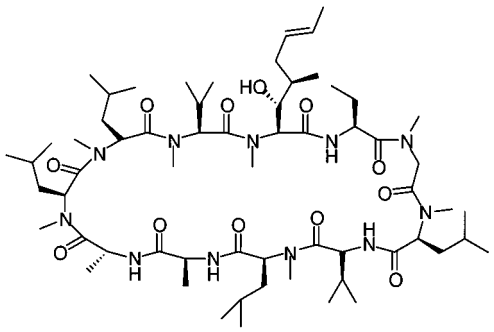
Cyclosporin-A (**19**) was isolated from a fungus, *Tolypocladium inflatum*, and was found to be a 5 nM inhibitor of PP2B.[53]



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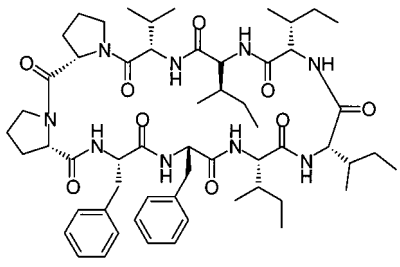
Cyclolineopeptide-A (CLA) (**20**) was isolated from linseed oil and was found to be a 5−10 μM inhibitor of PP2B.[54]



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4. Structural Features of Protein Phosphatase Inhibition

Crystal Structures, Molecular Modeling, Structure−Activity Data, and Inhibition of Protein Phosphatases. X-ray crystalography and mutagenesis studies have been utilized to determine the key structural features of the catalytic domain of protein phosphatases. Unfortunately, this direction of research has been hindered somewhat by the absence of the PP2A crystal structure, the elucidation of which has become the "holy grail" of phosphatase research. Nonetheless, by examining the crystal structure of the catalytic subunits of PP1[72,73] and PP2B,[74,75] when complexed with microcystin-LR, or tungstate (a phosphate analogue) and FK-506/FKBP, respectively, the overall structural features of the catalytic domains of the two enzymes appear to be very similar. Both have an NH_2-terminal subdomain containing two metal atoms embedded at the cores of a conserved phosphoesterase motif, DXH-$(X)_n$GDXXD$(X)_n$GNHD/E (n = 25), which is also found in other phosphoesterases.[76] The crystal structure of PP1 further suggests that catalysis involves nucleophilic attack by a metal-activated water molecule, and His-125 is likely to act as a general acid protonating the leaving group serine (or threonine). Furthermore, Asp-95 appears to be buried at the active site with a carbonyl oxygen forming a hydrogen bond with the N^{δ} atom of His-125; the location of Asp-95 suggests a catalytic role in confining the position of His-125 and stabilizing its protonated state.

Site directed mutagenesis studies of residues present in the active site of PP1 (summarized in Table 5)[77] support this, as the D95A mutant exhibited a large decrease in k_{cat} (>2000-fold) with no significant change in K_m. Interestingly, none of the acidic groove mutants (e.g., D220V, E275R) exhibited changes in sensitivity to any of the toxins. This is in contrast to mutations at the active site that showed a decreased sensitivity to microcystin, okadaic acid, and calyculin-A. These changes are consistent with that predicted by the PP1-microcystin-LR bound structure. For example, Arg-96 hydrogen bonded with the carboxylate group of the β-linked D-erythro-β-methylaspartic acid side-chain of microcystin-LR. The mutation R96A increased the IC_{50} value for microcystin-LR to 35 nM, an increase of >2000-fold. The contribution of the phenolic-hydroxyl was evident by a 35-fold increase in IC_{50} in the Y272F mutant. Notably, the Y272F mutant also caused a decrease in okadaic acid sensitivity (26-fold) and calyculin-A (107-fold), suggesting the importance of this residue in binding.

These results and other earlier mutagenesis studies highlight the roles of active site residues, not only in metal binding and catalysis, but also in binding to both toxins and the phosphorylated inhibitor protein, phospho-DARPP-32.[78] The results also suggest that acidic groove residues influence in some way the interaction of phospho-DARPP-32 with the active site of PP1. Mutation of any one of the metal binding ligands analyzed in these studies (Asp-64, His-66, Asp-92, N124, and His-248) resulted in a decreased affinity for metal ion that most likely explains the large decrease in k_{cat} values observed (Table 5). Interestingly, mutation of Tyr-272 had no effect on enzyme activity; this is somewhat surprising considering that this active site tyrosine is conserved in all of the protein phosphatases. However, it has been suggested that Tyr-272 in the β12/β13 loop is in a different position in native PP1 than that found in the bacterial preparation.[72]

The relative specificity of the various toxins for PP1 and PP2A appears to be explained in part by specific

Table 5. Kinetic Analysis and Inhibition of PP1 Mutants by Natural Toxins (Microcystin-LR, Okadaic Acid, and Calyculin-A) and S-DARPP-32

PP1 mutation	specific activity (units/mg)	K_m, μM	K_{cat}, s^{-1}	IC_{50} nM			
				microcystin-LR	okadaic acid	calyculin-A	S-DARPP-32
wild-type	34	10.6	39	0.02	45	0.45	115
N124D	2.0	19.8	3.1	200	>5000	>500	>1000
H248N	0.7	5.1	0.5	35	>5000	>500	>1000
D95A	0.03	5.8	0.02	-[a]	-	-	-
R96A	0.08	6.9	0.09	35	260	58	640
R221S	0.05	105.0	0.2	29	>5000	>500	960
D208A	8.0	23.3	13.3	1.9	>5000	>500	4.2
C127S	49	10.4	58	0.56	78	2.2	250
Y272F	26	5.0	14	0.80	1150	48	310
D220V	30	8.1	30	0.02	28	0.14	210
E256R	36	-	-	-	-	-	11
E275R	69	13.2	86	0.01	48	0.4	2.2
E252A:D253A	37	-	-	-	-	-	8.6
E252A:D253A:E256R	12	-	-	-	-	-	7.0

[a] Hyphen (-) = not determined.

interaction with variable amino acid side chains in the β12/β13loop.[79,80] However, it is less clear why PP2B is relatively resistant to inhibition, since all of the residues analyzed in the mutagenesis studies were conserved in PP2B as well as other protein phosphatases. Unlike protein kinases, where short peptides encompassing the phosphorylation site contain similar elements necessary for efficient enzyme binding, similar studies with the protein phosphatases have been much less informative.[81] Notably, the catalytic subunit of PP1 does not dephosphorylate short peptides, while PP2A does so with high efficiency, and PP2B is intermediate. With the exception of the R221S mutant, the affinity for phosphorylase was not affected by any of the mutants studied. Furthermore, the general substrate specificity of the acidic groove mutants was unchanged. These results raise the possibility that basic amino acids in phosphorylation sites of substrates such as phosphorylase may not bind to the acidic groove of PP1. In this respect, mutation of Asp-71, located in the COOH-terminal groove, has been found to reduce the affinity for phosphorylase.[78] Furthermore, the autoinhibitory region of PP2B, which is believed to be a competitive inhibitor, is located close to the hydrophobic groove, the active site, and the β12/β13loop, although there are not extensive contacts outside of the active site.[75]

A wealth of biochemical data now exists that defines PP2B as the immunosuppressant target of FK506 and Cyclosporin-A. Perhaps the most striking feature of the PP2B−FK506−FKBP12 ternary complex is the 22-residue B-subunit binding α-helix (BBH) that extends nearly 40 Å away from the surface of the phosphatase domain. The FKBP12−FK506 complex binds to PP2B at the base of the BBH, making contacts with the BBH, calcineurin-B, and the phosphatase domain of calcineurin-A. Several key residues, including Asp37, Arg42, His87, and Ile90, are identified as mediators of PP2B binding by FKBP12−FK506.[82−84] The principal site of interaction between FK506 and PP2B is a predominantly hydrophobic cleft located at the interface of calcineurin-B and BBH. In the ternary complex structure, FK506 is located 25 Å from the phosphatase active site and, as such, does not participate directly in PP2B inhibition. Instead FK506 mediates PP2B inhibition and the immunosuppression that follows by forming a composite effector surface with structural elements from FKBP12, which then recognize and bind tightly to PP2B. The bound FKBP12 physically blocks access by NF-At_p to PP2B's active site, not by a direct interaction with that site, but by virtue of its eclipsing position relative to it. Interestingly, the FKBP12-rapamycin complex does not inhibit PP2B, even at high concentration, and rapamycin acts as an antagonist of the immunosuppressive action of FK506.[82]

The PP2B active site, located on the surface of the phosphatase domain, is not in contact with any other component of the ternary complex. The nearest neighbor, FKBP12, is more than 10 Å removed. The phosphatase domain of the A-subunit forms an ellipsoid about 35 Å × 35 Å × 45 Å in size. The core of the domain consists of two mixed β-sheets, sheet 1 and sheet 2, that are flanked on one side by a mixed α/β structure and on the other by an all α-structure. The two central β-sheets form a distorted β-sandwich that contains an open and closed end. At the closed end of the β-sandwich, sheet 2 extends above sheet 1. The phosphatase active site is located above the closed end of the β-sandwich, formed by the convergence of several loops and by that portion of sheet 2 that extends above the β-sandwich. Active site residues are found in loops L2, L3, L4, and L2 and at the C-termini of strands β2 and β3. This broad, shallow active site is also consistent with observations that other serine/threonine protein phosphatases are able to catalyze the dephosphorylation of certain phosphotyrosine substrates as well as their natural substrates.[85]

PP2B's active site contains two metals, Zn^{2+} and Fe^{3+}, which agrees well with the crystal structure of purple-acid phosphatases (PAP) that also includes a Zn^{2+}/Fe^{3+} pair in its active site.[85] The Zn^{2+} is coordinated by the side chains Asp118(Oδ2), Asn150(Oδ1), His199(Nϵ2), and His281(Nδ1) and by a phosphate oxygen. This coordination sphere is identical to that of the Zn^{2+} in PAP. The preferred octahedral coordination of the Fe^{3+} is fully satisfied by a single bridging water or OH^-. This water would also be stabilized by its interaction with the carbonyl oxygen of His281.

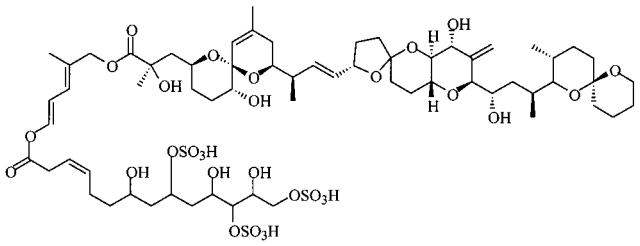
The likelihood of structural similarity between subfamilies of the serine/threonine protein phosphatase is further supported by a comparison of the PP2B active site residues with the results of mutagenesis studies carried out on the serine/threonine protein phosphatase from bacteriophage λ (λ-PPase). This study found that conservative mutations in λ-PPase of residues corre-

sponding to Asp90, His92, and Asp118 in calcineurin-A, all involved in metal coordination, resulted in a million-fold decrease in K_{cat}.[76]

Molecular Modeling. Since the early 1990s, molecular modeling approaches have been utilized in attempts to understand the key interaction of protein phosphatase inhibitors with these enzymes. There have been numerous modeling studies, including those of Lanaras,[86] Quinn,[87] Holmes,[88] and Armstrong.[89] Although elegantly simple, and addressing the known structure−activity relationship (SAR) data then available, the subsequent X-ray of microcystin-LR and PP1 cocrystallization and more complex modeling studies have suggested that this initial model was an oversimplification of the key features required for efficient binding. In particular, microcystin-LR is bound to PP1 with its cyclic core adopting a pseudo figure-eight conformation to maximize favorable contacts with the protein. More recently, these studies have culminated with the examination of the crystal's structure of PP1 and the modeled structure of PP2A.[90]

As a result, a pharmacophore model was developed that explains the selectivity of the existing natural toxins toward both PP1 and PP2A, and has been experimentally supported by the synthesis of potent and selective microcystin analogues.[91] This pharmacophore requires an acid moiety such as a carboxylate or phosphate, a hydrophobic tail, and a portion of the molecule that mimics a peptide binding to a phosphate recognition domain. Table 6 provides a summary of points of differential contact in PP1 (crystal) and PP2A (modeled) observed in these studies.

Structure−Activity Relationships (SAR). There has been limited SAR studies conducted on the okadaic acid class of compounds, largely due to the protracted and complex nature of their synthesis. However, there has been a significant amount of information gathered about key structural features of selected members of this class of compounds.



21

In the case of okadaic acid, SAR studies have shown that esterification, removal of the carboxylate, completely removes activity although, curiously, DTX-4 (**21**) bearing a sulfated polyol ester is only 10−50-fold less potent than okadaic acid;[92] ozonolysis of the C14−C15 double bond followed by reduction to two spiroketal fragments affords weak inhibitors of phosphorylated histone H1. The 7-hydroxyl is not important for binding; the 2-hydroxyl is crucial, modification or removal destroys the inhibitory activity. From these data it seems probable that the −COOH moiety is acting as a phosphoryl mimic, and the C2, C24, and C27 hydroxyls may be involved in intra- or intermolecular hydrogen bonding interactions.[93−95]

Table 6. Summary of the Contacts between the Okadaic Acid Class of Inhibitors, DARPP-32, and the Catalytic Site Residues of PP1 and PP2A[90] [a]

inhibitor	contacting group	PP1 residue(s)	PP2A residue(s)
cantharidic acid	7-O (bridging)	Tyr-272	Tyr-261, Tyr-263
	C3-COO-	Arg-96	Arg-87
	C3-COO-	Tyr-134	Tyr-123
	C2-Me	His-248	His-237
okadaic acid	C24-OH	Arg-221	Arg-210
	C27-OH	Arg-221	Arg-210
	C10-Me	Tyr-272	Tyr-263
	C13-Me	Phe-276	-
	C29-Me	Tyr-134	Tyr-123
	C2-OH	Arg-96	Arg-87
	C7-OH	-	Arg-264
	C2-Me	His-248	His-237
DARPP-32	**Arg-38**	**Glu-252**, Glu-256	Glu-242
	Arg-39	Asp-210, Asp-212	Asp-200, *Asp-201*
tautomycin	C25-Me	Tyr-272	Tyr-261, *Tyr-263*
	C19-Me	Phe-276	-
	C18-OH	Glu-275	-
	C7-Me	Tyr-134	Tyr-123
	C3-Me	Trp-206	Trp-196
	C1′(O)	Arg-221	Arg-210
	C22-OH	Arg-221	Arg-264
calyculin	C36-NMe_2	Glu-256	Glu-242
	C27-N	Asp-208	Asp-198
	C34-OH	Asp-210	Asp-200
	C35-OH	Asp-212	Asp-201
	C11-OH, C13-OH	Arg-221	Arg-210

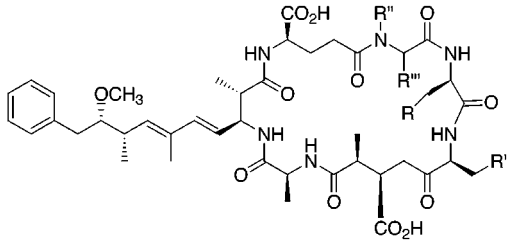
[a] Differential points of contact are highlighted in **boldface** type for PP1 and *italic* type for PP2A; highlighted residues are those which are either nonconserved or do not correspond to the same residues in both proteins.

Recently, Forsyth et al.[96] have developed a novel okadaic acid analogue lacking the hydrophobic domain which results in a significant decrease in activity against PP1 and PP2A.[96] At the same time, Forsyth et al. showed that the C1−C27 domain of 7-deoxy okadaic acid retains some activity against PP1, displaying an 800-fold decrease in potency, while exhibiting only a 50-fold decrease in PP2A inhibition. On the other hand, the C16−C38 fragment showed activity at neither PP1 nor PP2A (IC_{50}s > 1 mM).[97] These results highlight the importance of the C28−C38 hydrophobic domain for potent inhibition of PP1 and PP2A and that this domain is insufficient for activity. The hydrophobic domain is more crucial to the inhibition of PP1 than PP2A.

Inspection of tautomycin immediately reveals analogy to cantharidin since they both possess constrained anhydrides. Replacement of the C1′ substituent with a methyl ester renders the analogue inactive; a smilar trend is observed after deoxygenation or β-elimination of the C22-hydroxyl substituent.[97,98]

As a result of >60 known microcystins, and the solving of a Microcystin-LR/PP1 cocrystal, a reasonably detailed SAR profile is available.[91,99−106] Saturation of the dehydro-amino residue has little, if any, effect on inhibition; esterification of the Glu residue is detrimental to inhibition;[100] and the Adda side chain plays an important role in phosphatase inhibition by microcystins since hydrogenation or ozonolysis of the alkenoic positions attenuates activity as does the presence of a cis

Table 7. Comparison of the IC_{50} (nM) Values of Synthetic Microcystin LA and the Microcystin Analogues toward the Purified Catalytic Subunits of PP1 and PP2A[91]



inhibitor	R	R′	R″	R‴	inhibition (IC_{50} nM) PP1c	PP2Ac	PP1 selectivity
microcystin-LA (synthetic)	H	$CH(CH_3)_2$	CH_3	$=CH_2$	0.3	0.3	1
22	H	*c*-Hex	Me	CH_2	0.52	3.4	7
23	H	*i*-Pro	*c*-Hex	H	0.8	1.5	2
24	H	*i*-Pro	Me	H	0.8	1.5	2
25	NH_3^+	*i*-Pro	Me	CH_2	3	9	3

(rather than trans) geometry about the Δ^6 double bond.[101,102] However, the replacement of the C9 methoxy with a C9 acetoxy or C9 hydroxyl has no effect.[103−105]

Like the microcystins, the nodularins also have variable regions. In these instances, esterification of the isoglutamate carboxyl destroys activity, change of Adda's −OMe to −OH decreases toxicity 2-fold, changing D-MeAsp to D-Asp results in no loss of activity, and isomerisim of Adda's trisubstituted olefin from *E* to *Z* abolishes activity as does scission of the macrocycle between the L-Arg and Adda.[100]

Although sharing similar biological properties, important functional differences between the microcystins and nodularins have been identified. One difference is in the interaction with PP1c and PP2Ac. Although both toxins initially bind noncovalently and inhibit these enzymes, microcystin-LR, -LA, and -LL undergo a secondary time-dependent interaction with the phosphatase. In contrast, nodularin or motuporin do not bind covalently to PP1c or PP2Ac.[48]

Modeling studies of PP1 and PP2A (described above)[90] have resulted in the synthesis of four microcystin-LA analogues.[91] Table 7 summarizes the effect of these structural modifications on PP1 and PP2A inhibition. This work produced the most selective PP1 small peptidic molecule (**22**) inhibitor yet reported (7-fold). Importantly, this analogue also maintained high potency although it is not likely to be cell permeable, thus limiting its use. However, it does represent the first occurrence of computer assisted design of an inhibitor of either PP1 or PP2A, thus paving the way for further modifications, with greater potency and selectivity. Indeed of all the phosphatase inhibitors reported to date, only one, phosphatidic acid, can lay claim to being a specifc and selective inhibitor of PP1.[107]

Calyculins A−H are equally potent for both PP1 and PP2A. Of the few semisynthetic analogues that have been explored, decahydrocalyculin retains activity but the C11−C13 acetonide has an IC_{50} of >1 mM.[108,109]

In an attempt to adopt a combinatorial approach to the development of PP1 and PP2A inhibitors, Wipf et al. developed a basic pharmacophore (Figure 1).[110]

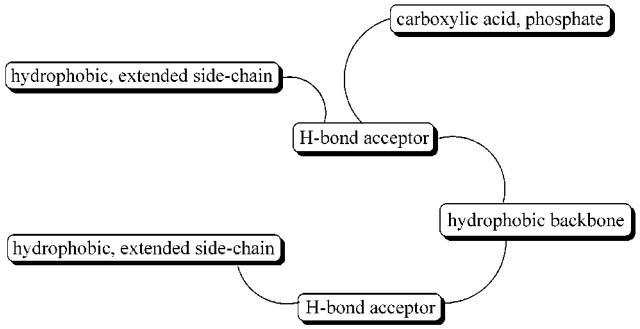
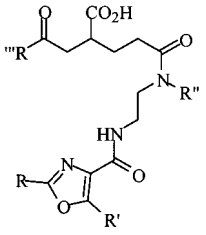


Figure 1. Basic pharmacophore developed by Wipf et al. for the development of PP1 and PP2A inhibitors.

As a result of the pharmacophore shown in Figure 1, a total of 18 analogues based on the parent compound **26** were synthesized.



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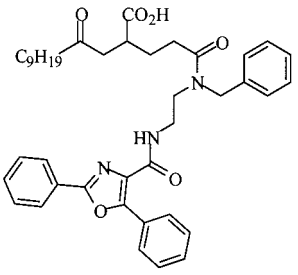
These studies gave rise to a number of analogues that inhibited PP1 and PP2A by >50% at 100 μM, which represents a moderate lead compound in this area. Analogues **27**−**29** showed the greatest inhibition of PP2A (IC_{50} <100 μM). Both **27** and **28** were cytotoxic to human breast carcinoma cells (MDA-MB-231) with an IC_{50} of <100 μM. Analogue **28** did not suppress cell proliferation significantly; however, **27** displayed classical concentration-dependent inhibition of proliferation in MDA-MB-231 cells. Flow cytometry indicated blockage at the G_1 checkpoint of the cell cycle. Interestingly, **29**, which blocked PP2A activity, did not appear to be cytotoxic. This lack of biological activity may be due to poor cell permeability.[110]



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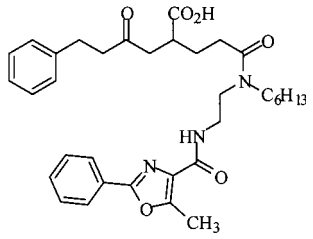

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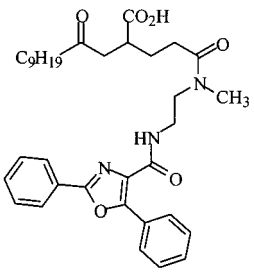

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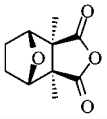
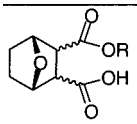
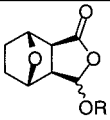
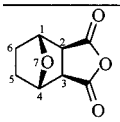
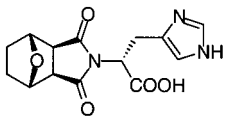
Table 8. Inhibition of Protein Phosphatases 1 and 2A by Anhydride Modified Cantharidin Analogues

Compound		R	Inhibition (IC_{50} μM)		PP2A
			PP1	PP2A	selectivity
	**10**		1.78	0.26	6.8
	**30**	R = Me	4.71	0.41	11.5
	31	R = Et	2.96	0.45	6.6
	32	R = Prop	4.82	0.47	10.3
	**33**	R = Me	>1000	>1000	-
	34	R = Et	746	55	13.6
	35	R = Prop	>1000	>1000	-
	**36**		1.98	0.37	5.5

Cantharidin, being by far the simplest inhibitor yet discovered, sparked a brief flurry of SAR reports. It is believed that for efficient binding the anhydride ring opens to the corresponding dicarboxylate, which sits inside the active site of both PP1 and PP2A.[111−118]

We and others had shown that any attempt to modify the anhydride of cantharidin which would prevent ring opening removes all inhibitory activity.[113] Modifications of the 7-O bridgehead are also not tolerated.[116] It has been suggested that the presence of the proximal methyl substituent (relative to the carboxylates) preorganizes or conformationally restricts the resultant conformations available to the diacid, thus increasing its inhibitory action.[48] This idea is reinforced by the observation that norcantharidin (**36**), the demethylated analogue, is 14-fold less active than the parent compound. Additionally, numerous studies have shown that substitition at the C1/C4 bridgeheads and the introduction of bulky groups at C5 are also delitereous to inhibition of PP2A. To date, there have been no published reports of any modifications that improve either the potency or the selectivity of cantharidin analogues. However, recent work from within our laboratory has indicated that marginal increases in PP2A selectivity via anhydride modified cantharidin analogues are possible, Table 8.[117]

Additionally, subsequent work from our laboratory has resulted in the development a new class of PP1 and PP2A inhibitors in which we have been able to maintain inhibition and modify norcantharidin's anhydride moiety. Of this new class of compounds, the cantharimides, only those possessing both a basic side chain and a free carboxylate, displayed potency, with the two most potent being the histidine containing **37** and **38**.[117]



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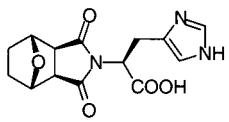

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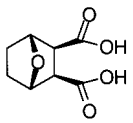
Tatlock et al.'s model of endothall (**39**) derivatives bound to PP2B's active site was generated by computational docking experiments and with information generated from a low-resolution cocrystal structure.[111]



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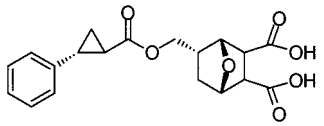
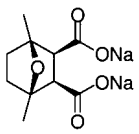
This model shows the dicarboxylic acid and the bridgehead oxygen acting as anchors, interacting with the catalytic site metals and surrounding residues. The 5-endo substitution appears to provide directionality into a region of the protein with potential for reasonable binding interactions. Attempts were made to mimic an interaction observed between Phe-470 of the autoinhibitory domain and the active site, in particular, the interaction between Phe-470 and a hydrophobic region of the active site specific to PP2B.[75] Of all the analogues synthesized, **40** showed both the highest inhibition and the greatest selectivity for interaction at PP2B (K_i(app) = 0.5 μM, PP2B:PP1 = 8), which represents a significant improvement upon endothall's inherent PP1 selectivity (PP1 IC_{50} = 4.0 μM; PP2B IC_{50} = 11.5 μM). It was suggested that the terminal phenyl ring of **40** binds in a similar position and orientation as Phe-70. This phenylalanine pocket is adjacent to PP2B Tyr-315. As it stands, the model suggests that Tyr-315 hydroxyl forms a hydrogen bond with the ester of the endothall-based ligands. With PP1, the equivalent residue is a Phe; PP2A, a Cys. The unconserved nature of this region was postulated as a reason for the differences in inhibitory effects, with no H-bonding opportunity present within PP1. No data was presented for the PP2A inhibitory effect; however, it is known that endothall analogues are potent PP2A inhibitors although substitutions at C5 are detrimental to PP2A inhibition. Consequently, moderate to good selectivity for PP2B versus PP2A is expected.



40

In a similar study, it was also reported that PP2B is tolerant of modifications at the 5-*endo* position of cantharidin analogues (it is believed that the dicarboylic acid form is the active species in the inhibition of protein phosphatases).[112] This work synthesized a series of skeletally modified cantharidin analogues, noting that methyl substitution at C1 and C4 effectively removed inhibitory effects at both PP1 and PP2A, while **41** still weakly inhibited PP2B.



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Reduction to one substituent at either C1 or C4 also reduced PP1 and PP2A inhibition, with PP2B again

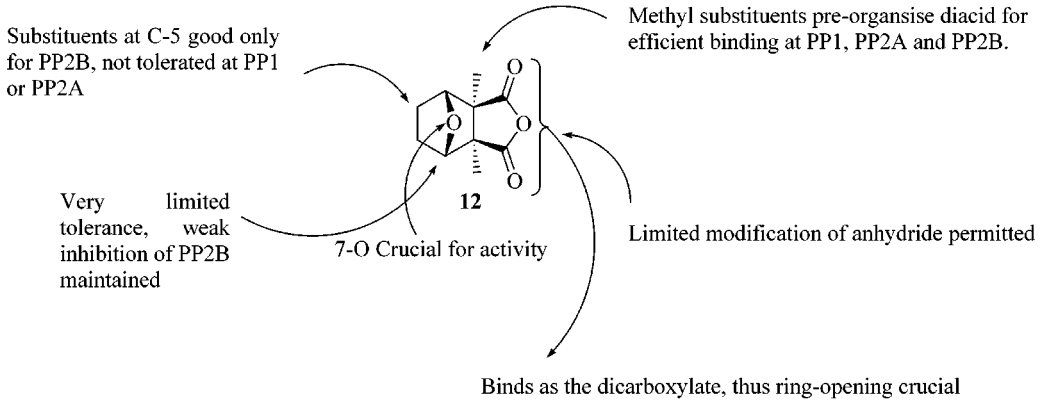
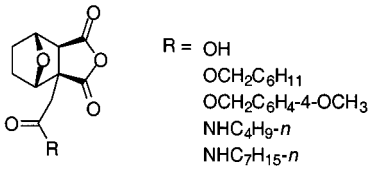


Figure 2. Key features and potential structural modifications of cantharidin and observed consequences for inhibition of the serine/threonine protein phosphatases. Note that cantharidin does not inhibit PP2C.

more tolerant of these modifications. PP2B is tolerant of substitution at C5. If both of these effects are additive, it appears that a small group at either C1 or C4 in conjunction with a larger group at C5, one that allows for a hydrogen bonding interaction with Tyr-315, may allow the development of more selective PP2B inhibitors. We can summarize the known SAR data pertaining to cantharidin analogues and the serine/threonine protein phosphatases as shown in Figure 2.

Laidley et al. reported the first skeletally modified cantharidin analogues (**42**) that maintained PP2A activity have been reported.[118]

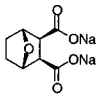
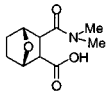


42

Others have also explored cantharidin analogues in an attempt to develop selective PP2B inhibitors,[123] by concentrating on modification of the anhydride. As a result, a series of dicarboxylic acid analogues were synthesized. No analogue showing PP2B selectivity was reported. However, the transcription of IL-2 in lymphocytes, which has been shown to be a PP2B-dependent process was examined.[119] The results of this study are shown in Table 9 and are compared with cyclosporin-A.

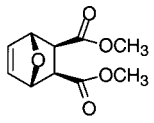
The difference in activity between **36**, **43**, and **44** may be due to the improved penetration of **44** into the cytosol. Also of note, **44** shows a 20-fold selectivity window between its inhibition of IL-2 transcription and release and its cellular toxicity. Previously, a possible immunosuppressive activity of **36**, indicating inhibition of lymphocyte proliferation and dose-dependent inhibition of a mixed lymphocyte reaction, was reported.[120] Both of these processes are strongly affected by PP2B inhibition, and even if other mechanisms are involved, the inhibition is consistent with the data reported by Pombo-Villar in Jurkat cells.[113] However, an in vivo study of cantharidin reported an increase in the spleen lymphocyte proliferation and of IL-2 and IL-1 production in mice. This may be due to the well-documented irritant and inflammatory properties of cantharidin.[121]

Table 9. Effect of Selected Cantharidin Analogues (and Cyclosporin-A) on IL-2 Production in Jurkat Cells[a]

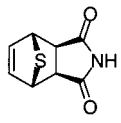
Compound		β-galactosidase IL-2 RGA IC_{50} μM	Inhibition of IL-2 secretion IC_{50} μM	β-galactosidase thymidine kinase RGA, IC_{50} μM
	**36**	>22, 100	ND	100
	**43**	>100, >100	ND	42
	**44**	4.7±1.2[b]	5	100
Cyclosporin-A	**19**	0.008±0.003[b]	0.007	>1

[a] IC_{50} values of individual experiments. [b] Mean and standard deviation of three experiments.

Although there has been limited success in the development of PP2B selective cantharidin analogues, we have recently reported the development of **45** and **46** as the first two PP1 (versus PP2A) selective cantharidin analogues, with PP1 selectivities of >40 and >30-fold and micromolar potencies observed.[122]



45



46

Considerable synthetic efforts have been required for the development of the SAR data presented herein. This effort has been assisted by recent advances in synthetic methodologies which have allowed the development of modular approaches to some of the complex toxins described herein, e.g., okadaic acid,[123−136] 7-deoxy okadaic acid,[137] tautomycin,[138−151] the microcystins/nodularins/motuporins,[152−170] the calyculins,[171−215] thyrsiferyl-23-acetate,[216,217] fostriecin,[218,219] cantharidins,[220−224] thus giving the medicinal chemist the ability to develop true structure−activity studies of these species and to develop combinatorial libraries utilizing the known toxins as lead compounds. The development of more specific and selective inhibitors will allow us to determine the roles of individual phosphatases in biological environments.

5. Therapeutic Use of Protein Phosphatase Inhibitors

The central role of protein phosphorylation/dephosphorylation in cellular regulation suggests that many disease states will involve perturbation in the balance between protein kinase and protein phosphatase activities. Given that many of the natural inhibitors of protein phosphatases lead to elevated protein phosphorylation levels within cells and in doing so alter associated processes, protein phosphatases should be considered attractive targets for novel drug design. The current acknowledgment that protein phosphatases are integrally associated with the regulation of many essential cell functions implies that they may be fundamental to the control of these functions. There are currently few examples of a direct association of protein phosphatases with disease, and clearly the credibility of these enzymes as targets for drug design requires substantial and continued validation. However, their role in modulating key cell processes that are altered in diseased states suggests that they are valid targets for the design of suitable intervention strategies.

Neurological Disorders. The major members of the PPP family are highly concentrated in brain and are fundamental components of the complex matrix of signaling pathways that control neuronal function.[225] PP1 is widely distributed in neurons and has multiple functions. Brain contains a specific homologue of I-1, DARPP-32, which is highly concentrated in dopaminergic neurons and has been linked with Parkinson's disease.[5] DARPP-32 is phosphorylated in response to dopaminergic receptor activation with consequential inhibition of PP1. Mice lacking DARPP-32 have considerably reduced responses to dopamine and display corresponding locomotor defects equivalent to those seen in Parkinson's disease.[226] Thus the targeted inhibition of PP1 in these neurons is a potential strategy for minimizing the symptoms associated with this disease.

PP1 is also integrally involved in two neuronal processes that have been studied as a model for learning and memory. Long-term potentiation (LTP) represents the activity-dependent enhancement of synaptic efficacy whereas long-term depression (LTD) is the activity-dependent reduction of synaptic efficacy. LTP and LTD represent the best experimental models for memory to date. The role of PP1 in these processes appears to predominantly involve I-1[227] although the targeting of PP1 to dendritic spines by spinophilin[9] and other unidentified proteins will clearly play a role in directing the specificity of PP1 action in the neuron. Stimuli that generate LTP were shown to facilitate PKA-mediated activation of I-1, hence suppressing PP1.[227] In contrast, stimuli that lead to LTD result in activation of PP2B which dephosphorylates and inactivates I-1.[228] In this way, I-1 and, by inference, PP1 activity appear to mediate the state of activation of the synapse. Specific inhibitors that modulate the activity of PP1 in this system have therefore the potential to alter the short- and long-term responsiveness of neuronal circuits. This may have therapeutic application in maximizing recovery following injury, enhancing learning, and ameliorating the negative effects of abnormal neuronal activity arising from hyper-responsive states in chronic pain or even drug abuse.

PP2B is highly enriched in hippocampal neurons and localized along dendritic spines, consistent with its proposed role in modulating LTP/LTD.[229,230] PP2B has limited substrates, and many of its roles have been attributed to dephosphorylation of DARPP-32 (see above) and activation of PP1. PP2B, because of its limited substrates, may therefore represent a better target for drug design (see immunosuppression below) in modulating the key functions that involve PP1 rather than PP1 itself, which has multiple actions. Moreover, other substrates, such as dynamin 1,[231] and the NMDA receptor coupled calcium channels play key roles in synaptic transmission and may therefore reflect a direct role for PP2B in learning and memory.[232] Overexpression of constitutively active PP2B in transgenic mice produced deficits in long-term memory and a constraint on LTP.[233] PP2B also appears to play a role in mediating the cytoskeletal rearrangements that occur with glutamate receptor induced neurotoxicity since immunosuppressive agents that inhibit PP2B block the cytoskeletal changes.[230]

Functions for PP2A in brain remain unclear with its role being inferred from its subcellular location and the substrates that it is targeted toward. PP2A has multiple locations in brain, with substantial proportions being directed toward the cell membrane[234] and cytoskeleton.[235] Of particular clinical interest is the recent implication that PP2A has a role in the etiology of Alzheimer's disease. A pool of PP2A is targeted toward neuronal microtubules[23] where it modulates the phosphorylation (and therefore function) of the key microtubule proteins, τ and MAP2. Expression of a specific inhibitor of PP2A (SV40 small t antigen) in neuronal cell lines leads to hyperphosphorylation of τ which has been proposed as a precipitating process in the neurodegeneration seen in Alzheimer's disease.[23] Moreover, studies have shown that activity of PP2A (and PP2B) toward the microtubule associated protein τ are reduced in Alzheimer's disease frontal cortex.[236] Clearly, in this case the role of the phosphatase is to restrain the levels of τ phosphorylation such that the therapeutic use of inhibitors will be counterproductive.

Metabolic Disorders. PP1 and PP2A are integrally involved in the mediation of insulin action on carbohydrate and lipid metabolism. More specifically, an activation of PP1 and inactivation of PP2A accompany insulin stimulation.[237] Activation of PP1 occurs through phosphorylation of its glycogen association regulatory subunit while PP2A inactivation appears to be through tyrosine phosphorylation of the catalytic subunit. Type-2 diabetes mellitus is characterized by a variation in response to insulin (insulin resistance) and any one of the molecules involved in the insulin signaling cascade is a potential target for therapeutic drug design. Indeed, studies of experimental models of diabetes demonstrated a reduction of insulin stimulated PP1 and a failure of insulin to inhibit PP2A activity.[238] Furthermore, polymorphisms in the PP1 glycogen targeting subunit have been associated with some forms of type 2 diabetes.[239] While major attention focuses on identifying the genetic mutations that lead to insulin resistance, compensating for the effect of such mutations is also a viable strategy. While the role of PP1 in the insulin signaling cascade is reasonably well established, clearly

drug mediated inhibition is most likely to enhance insulin resistance rather than compensate for it. A more likely target in the future would be to mimic the insulin-stimulated blockade of PP2A activity. However, the role of insulin-mediated PP2A inactivation in diabetes remains to be determined.

Respiratory Disease and Allied Disorders. Cystic fibrosis is a genetic disease causing defective transport of chloride ions in cells of the lung and pancreas. Inheritance of two of a range of defective alleles produces a defective chloride channel (CFTR) and subsequent overproduction of thick mucus. The CFTR protein is regulation by phosphorylation and dephosphorylation at multiple sites. The application of general protein phosphatase inhibitors resulted in activation of CFTR channels from cells expressing both wild type and, more importantly, a number of mutated CFTR channels.[240] While the recovery of function in mutated channels was restricted to those that were expressed at the cell membrane, this raises the possibility of phosphatase-inhibition targeted therapy for the treatment of at least some forms of CF. Recent studies show that while a number of phosphatases can dephosphorylate the CFTR channel, the PPM phosphatase PP2C is specifically associated with it.[241] Furthermore, inhibitors of the PPP family do not prevent inactivation of the channel, suggesting that PP2C is the predominant CFTR phosphatase.[242] PP2C may therefore be an important phosphatase to target for cystic fibrosis drug design.

The production of mucus in response to inflammatory mediators is a common feature of many respiratory disorders, including asthma. The human lung mast cell plays the primary initial role in the inflammatory response through the secretion of a number of inflammatory mediators. While phosphatase inhibitors are known to inhibit mast cell secretion, recent research indicates that PP2A is the principle phosphatase involved in this process and may be central to its control.[243] More specifically, a cytosolic pool of PP2A is transiently translocated to the cell membrane where it is activated. The translocation and activation is essential for secretion to occur, and altered rates of translocation are strongly correlated with altered rates of secretion. A detailed understanding of the mechanisms underlying this translocation and activation is required, and extended exposure of cells to phosphatase inhibitors has deleterious effects on cell viability. However, selective inhibitors targeted toward this specific pool of PP2A have great promise in preventing the abnormal inflammatory response seen in many respiratory diseases.

Immunosuppression. Potent and selective inhibitors of PP2B have attracted a great deal of their attention recently; given PP2B's crucial role in immunosuppression, this is not surprising.[244] It is known that the clinically useful immunosuppressive agents FK-506 and cyclosporin-A bind to intracellular receptor proteins and immunophilins and that this complex selectively inhibits PP2B leading to the suppression of T-cell proliferation. Interestingly in the absence of the immunophilin-FK-506 (cyclosporin) complex, neither FK-506 nor cyclosporin-A are inhibitors of PP2B.

Cyclolineopeptide-A (CLA, cyclo-[Pro_2-Val-Leu-Ile_2-Leu-Phe_2]) and its sulfonated analogue MC-172 were recently isolated from linseed oil and are PP2B inhibitors.[54] Both these species show similar immunosuppressant activity to cyclosporin-A and FK-506.[245] It is believed that the presence of CLA's pair of cis-linked proline residues is targeting the cyclosporin or FKBP-12 family of peptidyl-prolyl cis−trans isomerases (PPIases). Consequently, targeting of the PPIases may in turn give rise to a composite site with the ability to inhibit PP2B.

Both CLA and MC-172 were able to inhibit DNA synthesis at a concentration of $>3\ \mu$M, while a linear variant of CLA also inhibited DNA synthesis at higher concentrations. Thus sulfonation of CLA (giving MC-172) has no impact on inhibition but does improve solubility (a limiting factor in the use of cyclosporin-A). Additionally, CLA was able to inhibit dephosphorylation in the presence of cyclophilin A but not in the presence of FKBP-12. Furthermore, CLA inhibits PP2B phosphatase activity at $5-10\ \mu$M. MC-172 proved to be equally effective, reinforcing the fact that the sulfonyl group only improves solubility and does not interfere with PP2B inhibition.[54]

More interestingly, cyclosporin-A/cyclophilin and FK-506/FKBP-12 have quite different composite interfaces and interact with PP2B at slightly different sites.[246] It appears that the CLA/cyclophilin interface shares no obvious similarity with either the cyclosphorin-A or the FK-506- cyclophilin interface.

In addition to their immunosuppressive properties, FK-506 and cyclosporin-A have been reported to protect against NMDA neurotoxicity, suggesting that the mode of action may also involve PP2B inhibition.[247] In turn, the potential effect of FK-506 appears to involve functional inhibition of NOS (nitric oxide synthase) by blocking dephosphorylation. Thus NOS appears to be a PP2B substrate whose PKC-mediated phosphorylation is enhanced by FK-506. FK-506 may also inhibit the dephosphorylation of Ca^{2+}/calmodulin-dependent protein kinase or cyclic-nucleotide-dependent phosphorylation sites. The neuroprotective concentrations of FK-506 diminish NMDA enhancement of cGMP levels, which is NO-mediated.[248−250] The extent of cGMP inhibition brought about by FK-506 is about the same as the inhibition produced by 100 μM nitroarginine.[251]

Even though FK-506 is a potent inhibitor of PP2B, it is known to act at other sites such as steroid receptors, and it is possible that it mediates some of its biological effects via these receptors.[252] However, cyclosporin-A also protects against NMDA neurotoxicity, suggesting that the neuroprotective effect occurs via PP2B inhibition. Dawson et al. hypothesized that FK-506 neuroprotection also involves inhibition of NOS. The fact that FK-506 increases phosphorylation of a number of proteins indicates that other mechanisms may also play some role.[247]

It is possible that the neuroprotective effect of FK-506 (but not cyclosporin-A) may have an important clinical role to play. Since both FK-506 and cyclosporin-A are extensively used in transplant surgery as immunosuppressive agents and FK-506 crosses the blood-brain barrier (whereas cyclosporin-A does not), this explains the difference in their potential clinical use as neuroprotective agents.[253] In a liver transplant study encompassing a total of 28 patients in two groups of 14,

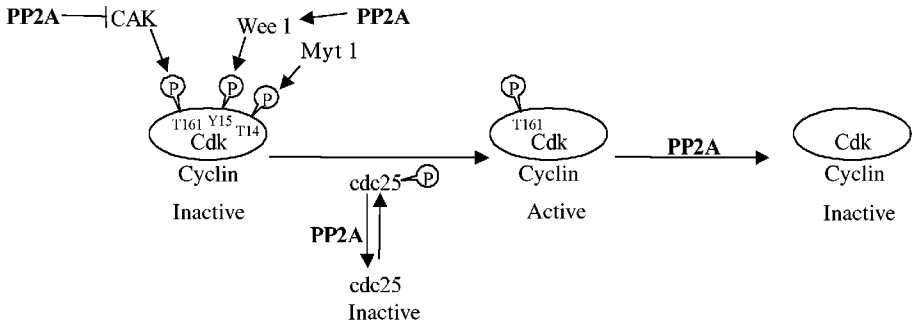


Figure 3. Cdk/cyclin activation.

7 of 14 patients who received cyclosporin-A showed cerebral ischaemia, whereas none of the 14 patients receiving FK-506 showed such an effect. This suggests that FK-506/immunophillin complexes (that inhibit PP2B) may have therapeutic potential.[254]

Cancer Therapy. Cancer is a disease that manifests as a result of excessive cell growth coupled with inadequate cell death (apoptosis). By understanding the role of protein phosphatases in the cell cycle and cell death, we can better assess the potential of targeting protein phosphatases as an anticancer therapy. Not surprisingly, an intricate phosphorylation network involving an interplay between kinases and phosphatases regulates the cell cycle.[255−260] While the role of kinases has been extensively examined, the function of phosphatases has lagged behind somewhat. In yeast and drosophila, mutations in the genes controlling PP1 and PP2A expression result in a variety of defects in mitosis, exemplifying the role of protein phosphatases in the cell cycle.[257] Yeast PP1 mutants are unable to complete anaphase successfully and unable to instigate chromosome segregation, while PP1 overexpression is lethal. In drosophila, PP1 mutants die at the larval-pupal boundary as a result of defective spindle organization, abnormal sister chromatid segregation, hyperploidy, and excessive chromosome condensation, as well as a delay in progression through mitosis. Yeast PP2A deficient mutants are not viable; however, mutants lacking one of the PP2A subunits display defects in cell septation and separation, and the cells become multinucleated, while in drosophila it leads to abnormal anaphase resolution.

The cell cycle involves four phases. The first gap (G_1) is followed by a phase of DNA synthesis (S-phase); this is followed by a second gap (G_2) which in turn is followed by mitosis (M) which produces two daughter cells in G_1. There are two major control points in the cell cycle, one late in G_1, and the other at the G_2/M boundary. The periodic association of different cyclins with different cyclin-dependent kinases (cdk) has been shown to drive different phases of the cell cycle; thus cdk4-cyclin D1 drives cells through mid G_1, cdk2-cyclin E drives cells in late G_1, cdk2-cyclin A controls entry into S-phase, and cdk1-cyclin B drives the G_2/M transition. The activities of these kinases depend on cyclin binding, phosphorylation state, and interactions with cdk-inhibitory proteins, such as p15, p16, p18, p19, p21, p27, and p57.[258]

(i) Cdk/Cyclin Activation. Cdk/cyclin complexes regulate the progression of cells through the cell cycle. The activation of which requires the phosphorylation of a conserved threonine residue (T161) by the cdk-activating kinase (CAK), as well as the removal of inhibitory phosphorylations. Wee1 and Myt1 kinases catalyze the addition of the inhibitory phosphorylation on tyrosine 15 (Y15) and threonine 14 (T14), respectively, while cdc25A, B, or C phosphatases catalyze their removal.[261] PP2A activates Wee1 via the removal of inhibitory phosphates[262] and inactivates cdc25s by removal of stimulatory phosphates.[256,263] PP2A can also dephosphorylate the conserved threonine residue (T161) directly inactivating the cdk/cyclin complex.[264] Collectively, PP2A inhibits the activation of cdk/cyclin complexes by inhibiting CAK activity, inhibiting cdc25 activity, stimulating Wee1 activity, and/or dephosphorylating the threonine residue on the cdk/cyclin complex (Figure 3). It is not surprising that PP2A inhibitors such as okadaic acid, fostriecin, and cantharidin have been shown to stimulate cell cycle progression and induce premature mitosis.[265−268] Similarly, overexpression of cdc25 in cells that lack Wee1 causes mitotic catastrophe characterized by multiple divisions without completion of DNA synthesis.[256]

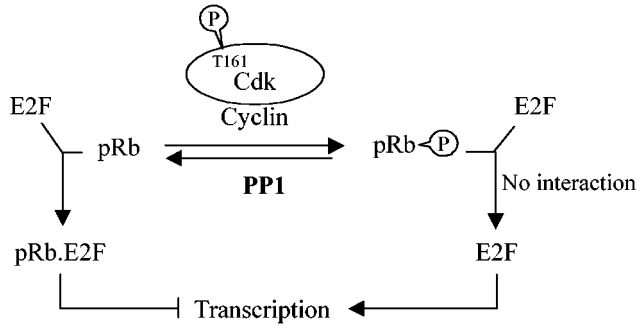


Figure 4. Phosphorylation of retinoblastoma protein.

(ii) G_1/S Phase Progression. During cell cycle progression, the G_1/S checkpoint is predominantly regulated by the cdk4/2-cyclin D/E complexes, which mediate their effects by phosphorylating and inactivating the tumor suppressor protein retinoblastoma (pRb). The phosphorylation of pRB prevents it from interacting with the S-phase transcription factor E2F, which controls the transcription of proteins needed for DNA synthesis. Therefore, the inactivation of pRB by phosphorylation permits entry into the S-phase of the cell cycle. This progression can be inhibited by the dephosphorylation of pRB by PP1 (Figure 4).[269−271] The phosphatase responsible appears to be PP1 rather than PP2A as the selective inhibition of PP1 by tautomycin maintained pRb in a highly phosphorylated state, while the selective inhibition of PP2A by okadaic acid and calyculin A decreased pRb phosphorylation.[271] However, others have shown okadaic acid to increase the pRb

phosphorylation.[267,272] This confusion arises due to the lack of truly specific protein phosphatase inhibitors and differing activities of protein phosphatases in cells. Nonetheless, PP1 is still described as a negative regulator of the cell cycle, the inhibition of which enhances cell cycle progression.

Clearly, additional mechanisms are required to control (stimulate or inhibit) movement through the cell cycle. Figure 4 illustrates such a mechanism where cdk's counteract the growth suppressive action of hypophosphorylated pRb by catalyzing the phosphorylation of pRb. This kinase family also inhibits PP1 activity via direct phosphorylation.[273] Throughout most of the cell cycle, including the G_2/M-phase, this state of pRb-hyperphosphorylation and PP1-inhibition is maintained. However, during late mitosis PP1 is reactivated, which in turn stimulates the growth suppressive action of pRb during the subsequent G_1-phase of the cell cycle.

(iii) Mitosis. While the late mitotic activation of PP1 is required for the dephosphorylation of pRb, it is also involved in the dephosphorylation of other mitotic proteins. Mitosis is a multi-stage process involving chromosome condensation, nuclear membrane disintegration, reorganization of cytoplasmic microtubules, spindle formation, chromatid separation, nuclear membrane reassembly, and cytokinesis, intricately controlled by protein phosphorylation. The multitude of reactions in the cell cycle controlled by phosphatase activity exemplifies the importance of this family of proteins in cell cycle progression.

In this context, in order for mitosis to occur the nuclear envelope disassembles and subsequently reassembles at the completion of mitosis, a process controlled by kinase and phosphatase action, respectively. The primary constituent of the nuclear envelope are lamins, which are members of the intermediate filament family, the phosphorylation status of which dictates the stability of the nuclear envelope. Hyperphosphorylation by protein kinase A, protein kinase C, and or cdk at the beginning of mitosis prevents the polymerization of lamin and results in disassembly, while hypophosphorylation by PP1 during the late anaphase/early telophase triggers reassembly.[274] In this context, phosphatase inhibition by okadaic acid has been shown to stimulate nuclear envelope breakdown in oocytes.[275]

Phosphorylation also mediates the stability of microtubules, which participate in the internal skeleton of the cell and the formation of mitotic spindles which mediates the segregation of chromosomes during anaphase of the cell cycle. The assembly and function of microtubules are dependent on their interaction with microtubule-associated proteins (MAPs), phosphorylation of which by MAP-kinases reduces this interaction. Conversely, protein phosphatases (predominantly PP1) dephosphorylate MAPs, resulting in increased microtubule assembly and stability.[276] The mechanism involved is not clear as the protein phosphatases may interact directly with the MAPs or indirectly by kinase inactivation. The latter may be the case as okadaic acid and calyculin A induce the selective breakdown of stable microtubules[277] and stimulate MAP kinase activity.[272]

Another important process during mitosis is the equatorial alignment of the DNA during the metaphase. In this reaction, the centromeric DNA binds to the DNA−protein complex known as the kinetochore which in turn binds to the microtubules of the mitotic spindle. The microtubule binding activity, but not the centromeric DNA binding activity, of the kinetochore complex is stimulated by PP1 and inhibited by opposing kinase activity.[278] *Saccharomyces cerevisiae* PP1 mutants exhibit low kinetochore-microtubule binding activity and a high frequency of chromosome loss as well as G_2/M arrest presumably as a result of activation of the spindle checkpoint.[278] Okadaic acid and microcystin also prevent kinetochore binding to spindle microtubules[278] and induce G_2/M arrest.[279]

The stability of other intermediate filament proteins including desmin and vimentin are also controlled by phosphorylation. At the cleavage furrow during cytokinesis, desmin and vimentin are phosphorylated by Rho kinase, which disrupts the filaments and allows for the segregation of intermediate filaments into daughter cells. Conversely, PP1 hyperphosphorylates these proteins allowing the filaments to repolymerize.[280] Okadaic acid and calyculin A have both been shown to phosphorylate desmin and vimentin filaments resulting in their collapse.[281]

(iv) Negative Regulators of the Cell Cycle. PP1 and PP2A are often classified as negative regulators of the cell cycle and therefore play an important role in cellular proliferation and differentiation. Such a conclusion is drawn from numerous observations where protein phosphatase inhibition results in enhanced cell growth including the stimulation of nonproliferating cells as well as tumor promotion.[267,272] For example, the treatment of quiescent hamster and human fibroblast cells with low doses of sodium *o*-vanadate or okadaic acid (<1 nM) allowed 30−40% of cells to progress from G_0-G_1 arrest to S-phase.[272] This was accompanied by phosphorylation of pRb and MAP-kinase proteins, as well as induction of the cdc2 (cdk-1) protein.[272] Similar concentrations of okadaic acid have also been shown to stimulate the growth of rat thyroid cells and involve increased cdk2 protein levels and increased pRb phosphorylation.[267] In these studies, the growth stimulatory effect of TSH in these cells was also potentiated in the presence of okadaic acid, as indicated by a 3.5-fold increase in DNA synthesis and enhanced G_1-S phase transition.[267] Okadaic acid[268,282] and fostriecin[265] have also been shown to induce premature mitosis and premature DNA condensation in various cell lines.

In addition to these natural toxins, other inhibitors of protein phosphatases including the small t viral antigen of the simian DNA virus (SV40) and the endogenous inhibitor SET have also been implicated in enhancing cell cycle progression. In this context, SV40 infection has been shown to stimulate DNA synthesis and growth of senescent cells[283] and is often used as a tool in the laboratory to transform nondividing cells. The intracellular target of the small t antigen is PP2A, binding of which inhibits PP2A activity, which stimulates MAP kinase and PKC ζ pathways, activates the transcriptional activator NF-κB, and induces cell proliferation.[284,285] The SET protein is a natural endogenous protein phosphatase inhibitor,[19] the overexpression of which has been observed in the childhood renal disease known as Wilm's tumor[286] and leukaemia.[19] The SET protein is normally expressed at greater levels in

the developing kidney than in the fully differentiated mature kidney; therefore, it is not surprising that abnormal expression of SET is found in this childhood tumor type but not in renal cell carcinoma, adult polycystic kidney disease, or in transitional cell carcinoma.[288]

In keeping with the role of PP1 and PP2A as negative regulators of the cell cycle, okadaic acid, microcystin, and calyculin A have been shown to induce tumor promotion in tissues intitiated with various agents such as dimethylbenzantharacene, methyl-nitro-nitroguanidine, or diethylnitrosoamine, in a manner different to that of phorbol esters.[287,288] These tumors show *ras* mutations and vimention hyperphosphorylation. In contrast, tautomycin which shares the same biochemical and biological activity of okadaic acid in vitro and in vivo fails to induce tumor promotion. Cantharidin also fails as both a tumor promotor and a carcinogen. In this context, cantharidin has been used topically (0.7%) as an anti-wart treatment since the early 1970s with no adverse effects.[289] Thus the role of protein phosphatases as negative regulators of the cell cycle is somewhat contentious and may only truly apply to certain stages of the cell cycle. For example, various phosphatase inhibitors (okadaic acid and cantharidin) stimulate cell cycle progression from G_1- to S-phase, but they also inhibit key mitotic events resulting in G_2 arrest.[290] Thus, the acceleration of the cell cycle appears to only occur during the first pass through the cell cycle and does not result in continued accelerated proliferation. This is further exemplified by the observation that PP2A stimulates the expression of cdk-1 and inhibits its action.[291]

In view of the role of PP1 and PP2A as negative regulators of the cell cycle it would appear that the use of protein phosphatase inhibitors as anticancer agents would be counterintuitive and stimulate cell growth rather than inhibit it. However, protein phosphatase inhibitors are lethal in a wide range of cancer cell types (detailed below) and have been clinically used to treat cancers. Although protein phosphatase inhibitors stimulate movement through the cell cycle, it is at an aberrant pace, and the cell reaches the mitotic stage before the cellular machinery required for mitosis is arranged and the cell dies.[268] Therefore, the use of PP1 and PP2A inhibitors in the treatment of cancers is likened to "fighting fire with fire".

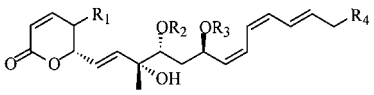
(v) Anticancer Activity of Protein Phosphatase Inhibitors. Clearly protein phosphatases are critical players in the cell cycle, the inhibition of which is a powerful therapeutic tool, particularly in the treatment of cell-cycle-dependent diseases such as malignant disease. In this context, the protein phosphatase inhibitors have been examined in the oncology arena with the potential exploitation as anticancer agents.

Both calyculin A and okadaic acid show strong anticancer activity in the murine leukaemia cell line, L1210,[64] and in human leukaemia cell lines including K562, HL60, and an adriamyosin-resistant variant of HL60 (HL60/ADR).[292] In these cell lines, calyculin A cytotoxicity (GI_{50} 1.75 μM), was 10 times greater than that of okadaic acid. Similarly in mouse neuroblastoma cells (N1E-115), the cytotoxicity of calyculin A (GI_{50} 2.6 nM, 24 h) was greater than that of okadaic acid (GI_{50} 31 nM, 24 h), cantharidin (GI_{50} 3.9 μM, 24 h), and mycrocystin LR (GI_{50} >5 μM, 24 h).[293] While the in vitro potency of these inhibitors dictates the intracellular cytotoxicity, membrane permeability is equally important. In this context, calyculin A and okadaic acid are both cell permeable; however, okadaic acid accumulates relatively slowly within cells, while mycrocystin-LR is not readily cell permeable.[293] Interestingly, cancer cell lines with induced resistance to okadaic acid show alterations in protein phosphatase activity, increased expression of P-glycoprotein (multi-drug resistance phenotype), and cross resistance to cisplatin and nitrogen mustard.[294,295] P-glycoprotein is a membrane associated pump that extrudes intracellular toxins and is characteristic of the multidrug resistance phenotype. While calyculin A and okadaic acid are clearly cytotoxic to cancer cell lines, the potency and toxicity is so great that they are not candidates for the in vivo arena. Notwithstanding this, the selectivity of these agents is invaluable in the elucidation of the intracellular role of protein phosphatases in malignant disease. Of the remaining natural protein phosphatase inhibitors, fostriecin and cantharidin hold the most promise as therapeutic agents in the treatment of malignant disease.

Fostriecin was initially screened by the NCI (U.S. National Cancer Institute) as a potential anticancer agent and entered phase I clinical trials on the basis of its ability to inhibit topoisomerase II (previously named CI-920). However, it was later shown to be a more potent protein phosphatase inhibitor; as a result, data pertaining to this action is lacking from preclinical studies. Nonetheless, in vitro fostriecin shows cytotoxic activity in a number of tumor cell lines including L1210 murine human leukaemia (GI_{50} of 0.21 μg/mL, 72 h exposure) and HCT-8 colon cells (GI_{50} 2.3 μg/mL, 96 h exposure).[296] In 53 cancer cell lines from the NCI cancer screen, fostriecin produced GI_{50} values in the low micromolar range (0.6−10 μM, 48 h) (Developmental Therapeutics Program, NCI). In other studies using the colony forming assay, fostriecin (10 μg/mL, continuous or 1 h) after 14−18 days in culture was active in 25 of 98 (26% response) human tumors of ovarian, breast, lung, and colon origin as well as Hodgkin's lymphoma and acute myelocytic leukaemia, with the greatest response occurring with the breast, ovarian, and lung cancers. Interestingly a lower dose of 1 mg/mL for 1 h produced a greater response (35%, 15 of 43 tumors) than a higher concentration of 10 μg/mL for 1 h (23%, 11 of 47 tumors) or 10 μg/mL as a continuous exposure (27%, 14 of 51 tumors).[297] When the antitumor response rate of fostriecin (35%) was tested against other anticancer drugs, it was shown to be among the most active drugs, at concentrations of 1/10 their maximal plasma level in humans, including BNCU, 33%; bisantrene, 38%; bleomycin, 13%; cis-platin, 9%; cyclophosphamide, 36%; doxorubicin, 18%; echinomycin, 0%; etoposide, 13%; 5-fluorouracil, 14%; hexamethylelamine, 60%; melphalan, 22%; methotrexate, 44%; MGBG, 17%; mitomycin C, 21%; mitoxantrone, 0%; vinblastine, 21%; vincristine, 25%.[295]

In vivo Fostriecin is also active against the L1210 and P388 leukaemias but had little effect against solid mouse tumors or against the human tumor xenografts including Ridgway osteogenic sarcoma, B16 melanoma,

Table 10. Structural Modifications and Antitumor Properties of Fostriecin Analogues in L1210 Leukaemia and HCT-8 Colon Cancer Cell Lines[299]



					GI_{50}[a] (μg/mL)	
compd	R_1	R_2	R_3	R_4	L1210	HCT-8
9	H	PO_3^{2-}	H	OH	0.21	2.3
47	H	PO_3^{2-}	H	H	0.22	1.5
48	OH	$PO3^{2-}$	H	OH	0.83	4.2
49	H[b]	PO_3^{2-}	H	OH	>1.0	56
50	H	H	H	OH	2.5	3.7

[a] GI_{50} concentration of drug that will reduce tumor cell growth to 50% of the control value. Adapted from ref 299. [b] Open lactone.

Lewis lung carcinoma, the CX-1 colon carcinoma, MX-1 mammary carcinoma, and LX-1 lung carcinoma, CD8F1 mammary adenocarcinoma, colon 38 adenocarcinoma, M5076 sarcoma, and 16/C mammary adenocarcinoma.[295] Fostriecin utilizes the reduced folate carrier system in order to enter the cells; it is also an inhibitor and irreversible inactivator of this carrier system. In this context, the inactivity of fostriecin in solid tumors in mice is believed to be caused by the lack of adequate transport, a phenomenon also observed for methotrexate in mice.[296,298]

Four phase 1 clinical trials with fostriecin have been initiated and coordinated by the NCI.[299] Only one has been completed, where fostriecin was administered iv over 60 min on days 1−5 at 4 week intervals (2−20 mg/m^2/day) in 20 patients.[300,301] No tumor responses were observed, but the plasma concentration reached in patients was insufficient to induce significant growth inhibition when tested in vitro; the maximum tolerated dose was not reached.[301] The instability and short half-life (0.36 h) of fostriecin may account for the lack of response (see below). The predominant toxicities were elevated liver transaminases (grade 4) and serum creatinine (grade 2), which were fully reversible. Other toxicities included nausea/vomiting (grade 1−2), fever, and mild fatigue, and almost no haematologic toxicity was observed.

Structurally modified analogues of fostriecin have been produced and screened for anticancer activity, Table 10. As a result of these studies, it has been found that the unsaturated lactone and phosphate ester moieties are required for anticancer activity, while ring hydroxylation or removal of the terminal hydroxyl group have only modest effects on activity.[296] The fostriecin structure is oxidatively unstable and loses much of its cellular activity within 30 min of incubation in culture medium,[265] and a similar half-life (0.36 h) was observed in patient plasma.[301] In patients a metabolite of fostriecin was also detected in the plasma and urine which was possibly the dephosphorylated analogue.[301]

Cantharidin is another protein phosphatase inhibitor with anticancer activity. Cantharidin has been shown to be active in cervical, tongue, ginival, neuroblastoma, bone, leukaemia, ovarian, and colon cancer cell lines cells.[114,290,293,302−304] Such growth inhibitory assays produced GI_{50} values in the low micromolar range of 1.5 to 15 μM. Cantharidin is also active in ovarian, melanoma, and epidermoid carcinoma (originally from primary tumor of the larynx) biopsies, but it is less active in cancer biopsies of lung, adenocarcinoma, pancreas, breast, cervix, mesothelioma, or sarcoma tumors.[305] Importantly, cantharidin in contrast to okadaic acid is not a substrate for P-glycoprotein.[306] Cells expressing this multidrug-resistant phenotype (LoVo adenocarcinoma cells made resistant to doxorubicin) were resistant to okadaic acid and 2-fold more sensitive to cantharidin when compared with the parental LoVo cells.[306]

The first recorded clinical use of cantharidin as an in vivo anticancer agent was in 1264 AD.[303] Cantharidin has been used in China for the treatment of hepatoma and oesophageal carcinoma.[303] Interestingly, clinical trials involving cantharidin have shown this agent to stimulate the bone marrow production of white cells. This is in contrast to most other anticancer drugs that have the unwanted side effect of inducing myelosuppression, which leaves the patients more susceptible to infection. Although cantharidin is cytotoxic to cancer cells and stimulatory on the bone marrow, the urinary toxicity of this drug has prevented its use in mainstream oncology.

Norcantharidin, the demethylated analogue of cantharidin, displays similar protein phosphatase inhibition (IC_{50} PP1 = 2.0 μM, PP2A = 0.4 μM)[114] to cantharidin and also possesses anticancer activity; however, the urinary toxicity characteristic of cantharidin treatment is absent.[303] Norcantharidin is active in vitro against HeLa, CaEs-17, Bel-7402, SMMC-7721 human hepatoma, CHO Chinese hamster ovarian and HEP-2 human epidermoid laryngocarcinoma, HL60 human leukaemia, and K-562 human leukaemia cell lines.[303] In our own laboratory, norcantharidin induced GI_{50} values ranging from 13 to 47 μM after 72 h exposure, which on average was 4-fold greater than that for cantharidin.[114,290] Interestingly, methotrexate- and piritrexim-resistant K-562 leukaemia cells were more sensitive to norcantharidin than the parental K-562 cells,[307] indicating that norcantharidin-induced cytotoxicity was not mediated by inhibition of dihydrofolate reductase. Furthermore, norcantharidin was still active against piritrexim-resistant cells which exhibit the multidrug-resistant phenotype, indicating that norcantharidin is also not a substrate for the P-glycoprotein transporter system.[307]

Norcantharidin has been used in vivo in the treatment of primary hepatoma, esophageal, gastric, and cardia carcinomas.[303] Norcantharidin increased the mean survival time of 285 reported cases with primary hepatoma from 4.7 to 11.1 months, and the 1 year survival rate from 17 to 30%, as compared to 102 patients treated with conventional chemotherapy (5FU, hydroxycamptotherine, vincristine, thiophosphoramide, and mitomycin).[303] As with cantharidin, norcantharidin not only failed to induce myelosuppression but also induced haemopoiesis via bone marrow stimulation.[308] This in vivo response was transient, lasting one week with the white blood count returning to normal following chronic administration. Interestingly, in mice, norcantharidin has been shown to block the leukopenia caused by cyclophosphamide;[303] therefore, such an agent may antagonize myelosuppression induced by other chemotherapeutic agents.

Other cantharidin analogues have been synthesized by Wang and screened for anticancer activity, and

Table 11. GI_{50}[a] Values of Tumor Cell Lines after 72 h Continuous Exposure To Test Compounds **10**, **30**−**36**

tumor type cell line	GI_{50} values, μM							
	10	**30**	**31**	**32**	**33**	**34**	**35**	**36**
A2780[b]	10 ± 2	100 ± 10	185 ± 5	110 ± 10	538 ± 83	100 ± 20	375 ± 176	50 ± 0
ADDP[b]	11 ± 1.2	180 ± 8	330 ± 39	315 ± 65	323 ± 40	107 ± 9	535 ± 120	47 ± 3
143B[c]	10 ± 1.2	118 ± 8	437 ± 37	450 ± 50	>1000	103 ± 17	>1000	43 ± 9
HCT116[d]	9 ± 1	76 ± 14	266 ± 9	75 ± 5	143 ± 23	80 ± 17	195 ± 5	24 ± 4
HT29[d]	6.4 ± 0.7	105 ± 5	243 ± 39	15 ± 4.5	28 ± 1	26 ± 8	41 ± 11	33 ± 7

[a] IC_{50} is the concentration that induces 50% growth inhibition compared with untreated control cells. [b] Ovarian. [c] Osteosarcoma. [d] Colon.

pharmacokinetics including disodium cantharidate, disodium norcantharidate, dehydronorcantharidin, hydrocantharidimide, and methylcantharidimide, all of which show anticancer activity.[303] The ease with which norcantharidin is synthesized compared with cantharidin renders this family of compounds more favorable for development as anticancer agents with potentially fewer toxic side effects than the parent compound. In this regard we have recently reported the development of a series of cantharidin analogues (**30**−**35**) that show promise as selective colon tumor anticancer agents, particularly **32**, Table 11.[114]

Not surprisingly, the in vitro protein phosphatase inhibitory action of these agents does not consistently parallel the in vivo cytotoxicity for various reasons, including differing protein phosphatase selectivity, membrane permeability, stability, and the potential for other intracellular targets. However, the ability of particular analogues to selectively target the colon tumor types is very intriguing and clearly warrants further development.

(vi) Drug Combination Therapy. While the synthesis and development of new single agent anticancer therapies has been the normal practice for organic chemists/oncologists in the past, it is clearly evident that the chemotherapy of the future will undoubtedly be based upon combinatory treatment. For example, the ability of protein phosphatase inhibitors to force cells prematurely through the cell cycle is intriguing when such agents are coupled with an agent that induces DNA damage. Such a synergistic response may force DNA damaged cells pass cell cycle checkpoints and directly into lethal mitosis. Such therapies may also target the more aggressive tumor types, which inherently have alterations in cell cycle checkpoint control including colon and nonsmall cell lung cancers.

A number of protein phosphatase inhibitors including okadaic acid, fostriecin, and cantharidin have all been shown to accelerate movement through the cell cycle and/or to abrogate either the G_1 or G_2 checkpoints, forcing cells prematurely into mitosis.[265,268,272,282,309] We have also shown cantharidin (25 μM) to accelerate movement of L1210 leukaemia cells through the G_1/S interface as evident by an increase in the S-phase population from 28 to 41% within 6 h; by 24 h G_2/M arrest predominated,[290,310] and a similar response was observed in HCT116 and HT29 colon cells.

Other agents have been shown to abrogate cell cycle checkpoints induced in response to DNA damage including nucleotide analogues (caffeine) and, interestingly, protein kinase inhibitors such as UCN-01. This kinase inhibitor has entered clinical trials as a combination treatment with radiation. Calyculin A (2.5−20 nM, 30 min) has been shown to substantially increase the cytotoxicity of radiation treated BHK21 fibroblast cells as determined by the colony forming assay.[311] As a single agent, this regime of calyculin A treatment was relatively nontoxic. The mechanism of this radiosensitization is not clear; however, calyculin A induces chromatin condensation, an event known to influence radiosensitivity. Interestingly, okadaic acid was less effective than calyculin A at enhancing X-ray killing.[266] In a final comment, Wilm's tumor (childhood renal disease) is characterized by the overexpression of the endogenous protein phosphatase inhibitor SET and interestingly this tumor type is one of only a few tumors that can essentially be cured by chemotherapy. This begs the question, is the enhanced sensitivity to chemotherapy a result of inherently low protein phosphatase activity and if so can this be mimicked in other tumor types using exogenous protein phosphatase inhibitors?

Apoptotic Cell Death. The role of protein phosphatases in apoptotic cell death is as intricate as that for cell growth. Many, if not most, protein phosphatase inhibitors including okadaic acid,[312] calyculin A,[312] thyrsiferyl 23-acetate,[312] tautomycin,[312] cantharidin,[306] norcantharidin,[314] nodularin,[315] microcystin-LR,[316] cyclosporin A,[316] FK506,[316] and viral proteins (E4orf4)[317,318] have all been shown to induce apoptotic cell death. Although these agents undoubtedly induce cell death via protein phosphatase inhibition, which results in a multitude of cellular stresses, the downstream mechanisms involved are not known. Such studies are confounded by the observation that many of these protein phosphatase inhibitors also inhibit or reduce the susceptibility of cells to apoptosis including okadaic acid, calyculin A, cantharidin, FK506, and cyclosporin A.[319−321]

The ability of protein phosphatase inhibitors to prevent apoptosis and maintain cell survival appears to involve alterations in the phosphorylation of key apoptotic proteins. For example, the serine/threonine hyperphosphorylation of proteins such as pRb,[322] bcl-xl, and BAD (serine 112, 136)[319,323,324] are linked with decreased apoptosis (cell survival) while their dephosphorylation is linked with apoptotic cell death. The activation of the antiapoptotic protein bcl-2 also appears to be controlled by serine/threonine phosphorylation; however, some studies have shown inactivation of bcl-2 by dephosphorylation[325] while others have shown inactivation of bcl-2 by phosphorylation.[326] Furthermore, bcl-2 overexpression prevents apoptosis induced by okadaic acid[327] but not nodularin or microcystin.[315] Similar inconsistencies are observed for cyclosporin A where its ability to inhibit apoptosis is reported to involve the inhibition of the mitochondrial permeability transition reaction, a key feature of apoptotic cell death.[328] However, in our own studies, cyclosporin A failed to influence this reaction induced by the antimetabolite agent, Thymitaq.[329] Clearly the role of protein

phosphatase activity and apoptotic cell death is intricate, with many of these responses dependent upon the cell line understudy, the type of agent used to induce apoptosis, not to mention the type and concentration of the protein phosphatase inhibitor examined.

6. Conclusions and Future Directions

In this perspective review we dare to suggest that serine and threonine protein phosphatases are a novel medicinal target for the treatment of disease. The identification of new targets and the development of new therapies with appropriate therapeutic indices is an arduous task. The therapeutic index defines the ratio between the drug dose that achieves an effective disease response versus that dose that induces toxicity. Clearly the goal is to obtain a therapeutic index that is greater than currently obtainable in the clinic with conventional therapies. In this perspective review we have outlined what is currently known of serine and threonine protein phosphatases and their respective inhibitors. We have identified various lead compounds which are potentially amenable to further development and which may ultimately be exploited in the treatment of Parkinson's disease, neuronal damage, diabetes, cystic fibrosis, immunological disorders, and cancer. The identification of these lead compounds will form the basis of more rational mechanism-based design strategies, which will serve to fine-tune their biologic activity.

This direction of research is clearly in its infancy. To drive this area forward, various basic hurdles need to be identified and overcome. For the medicinal chemist this includes the complete characterization of the crystal structure of each protein phosphatase so that unique binding interactions can be identified. Until now, only PP1, PP2B, and PP2C have been characterized. The characterization of other family members will allow the development of specific and selective inhibitors of these targets enzymes, revolutionalizing studies into protein phosphatase activity. Equally challenging for the biochemist and clinician will be the identification and characterization of the role of protein phosphatases in biological systems and disease states. Numerous pitfalls threaten the progress of developing new therapeutics including poor solubility, unknown metabolism, excessive early toxicity, ineffective route or schedule of administration, inappropriate formulation, long-term unpredicted toxicities, delays in clinical assessment, and drug resistance. Nonetheless, newer agents afford novel ways to mechanistically target diseases; the challenge cannot be ignored.

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References

(1) International Human Genome Sequencing Consortium. Initial sequencing and analysis of the genome. *Nature* **2001**, *409*, 860.
(2) Dombradi, V. Personal communication.
(3) Barford, D.; Das, A. K.; Egloff, M. P. The structure and mechanism of protein phosphatases: Insights into catalysis and regulation. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 133−164.
(4) Huang, F. L.; Glinsmann, W. H. Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. *Eur. J. Biochem.* **1976**, *70*, 419−426.
(5) Hemmings, H. C. J.; Greengard, P.; Tung, H. Y.; Cohen, P. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature* **1984**, *310*, 503−505.
(6) Johnson, D. F.; Moorhead, G.; Caudwell, F. B.; Cohen, P.; Chen, Y. H.; Chen, M. X.; Cohen, P. T. W. Identification of protein phosphatase-1-binding domains on the glycogen and myofibrilar targetting subunits. *Eur. J. Biochem.* **1996**, *239*, 317−325.
(7) Beullens, M.; Van Eynde, A.; Stalmans, W.; Bollen, M. The isolation of novel inhibitory polypeptides of protein phosphatase 1 from bovine thymus nuclei. *J. Biol. Chem.* **1992**, *267*, 16538−16544.
(8) Beullens, M.; Stalmans, W.; Bollen, M. Characterization of a ribosomal inhibitory polypeptide of protein phosphatase-1 from rat liver. *Eur. J. Biochem.* **1996**, *239*, 183−189.
(9) Allen, P. B.; Ouimet, C. C.; Greengard, P. Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9956−9961.
(10) Westphal, R. S.; Tavalin, S. J.; Lin, J. W.; Alto, N. M.; Fraser, I. D. C.; Langeberg, L. K.; Sheng, M.; Scott, J. D. Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. *Science* **1999**, *285*, 93−96.
(11) Allen, P. B.; Kwon, Y. G.; Nairn, A. C.; Greengard, P. Isolation and characterization of PNUTS, a putative protein phosphatase 1 nuclear targeting subunit. *J. Biol. Chem.* **1998**, *273*, 4089−4095.
(12) Printen, J. A.; Brady, M. J.; Saltiel, A. R. PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. *Science* **1997**, *275*, 1475−1478.
(13) Schillace, R. V.; Scott, J. D. Association of the type 1 phosphatase with the A-kinase anchoring protein AKAP220. *Curr. Biol.* **2000**, *9*, 321−324.
(14) Tu, J.; Song, W. J.; Carlson, M. Protein phosphatase type 1 interacts with proteins required for meiosis and other cellular processes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **1996**, *16*, 4199−4206.

(15) Helps, N. R.; Barker, H. M.; Elledge, S. J.; Cohen, P. T. W. Protein phosphatase 1 interacts with p53BP2, a protein which binds to the tumour suppressor p53. *FEBS Lett.* **1995**, *377*, 295−300.
(16) Hirano, K.; Erdodi, F.; Patton, J. G.; Hartshorne, D. J. Interaction of protein phosphatase type 1 with a splicing factor. *FEBS Lett.* **1996**, *389*, 191−194.
(17) Mumby, M. C.; Walter, G. Protein phosphatases and DNA tumor viruses: transformation through the back door? *Cell Regul.* **1991**, *2*, 589−598.
(18) Mullane, K. P.; Ratnofsky, M.; Cullere, X.; Schaffhausen, B. Signaling from polyomavirus middle T and small T defines different roles for protein phosphatase 2A. *Mol. Cell. Biol.* **1998**, *18*, 7556−7564.
(19) Li, M.; Makkinje, A.; Damuni, Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J. Biol. Chem.* **1996**, *271*, 11059−11062.
(20) Lechward, K.; Zolnierowicz, S.; Hemmings, B. A. Eukaryotic translation termination factor 1 associates with protein phosphatase 2A and targets it to ribosomes. *Biochemistry* **1999**, *64*, 1373−1381.
(21) Tung, H. Y. L.; De Rocquigny, H.; Zhao, L. J.; Cayla, X.; Rogues, B. P.; Ozon, R. Direct activation of protein phosphatase-2A(O) by HIV-1 encoded protein complex NCp7. *FEBS Lett.* **1997**, *401*, 197−201.
(22) Okamoto, K.; Kamibayashi, C.; Serrano, M.; Prives, C.; Mumby, M. C.; Beach, D. p-53 Dependent association between cyclin G and the B′ subunit of protein phosphatase 2A. *Mol. Cell. Biol.* **1996**, *16*, 6593−6602.
(23) Sontag, E.; Nunbhakdi-Craig, V.; Lee, G.; Bloom, G. S.; Mumby, M. C. Regulation of the phosphorylation state and microtubule-binding activity of tau by protein phosphatase 2A. *Neuron* **1996**, *17*, 1201−1207.
(24) Voorhoeve, P. M.; Hijmans, E. M.; Bernards, R. Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. *Oncogene* **1999**, *18*, 515−524.
(25) Westphal, R. S.; Anderson, K. A.; Means, A. R.; Wadzinski, B. E. A signaling complex of Ca^{2+}-calmodulin-dependent protein kinase IV and protein phosphatase 2A. *Science* **1998**, *280*, 1258−1261.
(26) Ricciarelli, R.; Azzi, A. Regulation of recombinant PKC-alpha activity by protein phosphatase 1 and protein phosphatase 2A. *Arch. Biochem. Biophys.* **1998**, *355*, 197−200.
(27) Westphal, R. S.; Coffee, R. L. J.; Marotta, A.; Pelech, S. L.; Wadzinski, B. E. Identification of kinase-phosphatase signaling modules composed of p70 S6 kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. *J. Biol. Chem.* **1999**, *274*, 687−692.
(28) Li, M.; Makkinje, A.; Damuni, Z. Molecular identification of I_1^{PP2A}, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry* **1996**, *35*, 6998−7002.
(29) Adler, H. T.; Nallaseth, F. S.; Walter, G.; Tkachuk, D. C. HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A. *J. Biol. Chem.* **1997**, *272*, 28407−28414.
(30) Seeling, J. M.; Miller, J. R.; Gil, R.; Moon, R. T.; White, R.; Virshup, D. M. Regulation of beta-catenin signaliing by the B56 subunit of protein phosphatase 2A. *Science* **1999**, *283*, 2089−2091.
(31) Hsu, W.; Zeng, L.; Costantini, F. Identification of a domain of Axin that binds to the serine/threonine protein phosphatase 2A and a self-binding domain. *J Biol. Chem.* **1999**, *274*, 3439−3445.
(32) Virshup, D. M. Protein Phosphatase 2A: a panolpy of enzymes. *Curr. Opin. Cell Biol.* **2000**, *12*, 180−185.
(33) Nanahoshi, M.; Tsujishita, Y.; Tokunaga, C.; Inui, S.; Sakaguchi, N.; Hara, K.; Yonezawa, K. Alpha4 protein as a common regulator of type 2A-related serine/threonine protein phosphatases. *FEBS Lett.* **1999**, *446*, 108−112.
(34) Janssens, V.; Van Hoof, C.; Merlevede, W.; Goris, J. PTPA regulating PP2A as a dual specificity phosphatase. *Methods Mol. Biol.* **1998**, *93*, 103−115.
(35) Endo, S.; Suzuki, M.; Sumi, M.; Nairn, A. C.; Morita, R.; Yamakawa, K.; Greengard, P.; Ito, M. Molecular identification of human G-substrate, a possible downstream component of the cGMP-dependent protein kinase cascade in cerebellar Purkinje cells. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 2467−2472.
(36) Klee, C. B.; Ren, H.; Wang, X. Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J. Biol. Chem.* **1998**, *273*, 13367−13370.
(37) Coghlan, V. M.; Perrino, B. A.; Howard, M.; Langeberg, L. K.; Hicks, J. B.; Gallatin, W. M.; Scott, J. D. Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. *Science* **1995**, *267*, 108−111.
(38) Snyder, S. H.; Lai, M. M.; Burnett, P. E. Immunophilins in the nervous system. *Neuron* **1998**, *21*, 283−294.
(39) Lai, M. M.; Burnett, P. E.; Wolosker, H.; Blackshaw, S.; Snyder, S. H. Cain, a novel physiologic protein inhibitor of calcineurin. *J. Biol. Chem.* **1998**, *273*, 18325−18331.
(40) Collins, E.; Sim, A. T. R. Regulation of neuronal protein phosphatases 1 and 2A during development. *Methods Mol. Biol.* **1998**, *93*, 79−102.
(41) Zhao, S.; Lee, E. Y. C. A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. *J. Biol. Chem.* **1997**, *272*, 28368−28372.
(42) Kretsinger, R. H.; Nockolds, C. E. Carp muscle calcium-binding protein. II. Structure determination and general description. *J. Biol. Chem.* **1973**, *248*, 3313−3326.
(43) Takekawa, M.; Maida, T.; Saito, H. Protein phosphatase 2C-alpha inhibits the human stress-responsive p38 and JNK MAPK pathways. *EMBO J.* **1998**, *17*, 4744−4752.
(44) Shenolikar, S.; Nairn, A. C. Model systems in signal transduction. *Adv. Second Messenger Phosphoprotein Res.* **1991**, *23*, 1−21.
(45) Shenolikar, S. Protein serine/threonine phosphatases − new avenues for cell regulation. *Annu. Rev. Biochem.* **1994**, *10*, 453−508.
(46) Hubbard, M. J.; Cohen, P. On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem. Sci.* **1993**, *18*, 172−177.
(47) Faux, M. C.; Scott, J. D. More on target with protein phosphorylation: conferring specificity by location. *Trends Biochem. Sci.* **1996**, *21*, 312−315.
(48) Sheppeck, J. E.; Gauss, C. M.; Chamberlin, A. R. Inhibition of the Ser-Thr phosphatases PP1 and PP2A by naturally occurring toxins. *Bioorg. Med. Chem.* **1997**, *5*, 1739−1750.
(49) Li, Y. M.; Mackintosh, C.; Casida, J. E. Protein phosphatase 2A and its [^{3}H]Cantharidin/[^{3}H]endothall thioanhydride binding site. Inhibitor specificity of cantharidin and ATP analogues. *Biochem. Pharmacol.* **1993**, *46*, 1435−1443.
(50) Murray, L.; Sim, A. T. R.; Rostas, J. A. P.; Capon, R. J. Isopalinurin − A mild protein phosphatase inhibitor from a southern Australian marine sponge, Dysidea Sp. *Aust. J. Chem.* **1993**, *46*, 1291−1294.
(51) Capon, R. J.; Rooney, F.; Murray, L. M.; Collins, E.; Sim, A. T. R.; Rostas, J. A. P.; Butler, M. S.; Carroll, A. R. Dragmacidins: New protein phosphatase inhibitors from a Southern Australian deep-water marine sponge, *Spongosorites Sp. J. Nat. Prod.* **1998**, *61*, 660−662.
(52) Born, T. L.; Myers, J. K.; Widlanski, T. S.; Rusnak, F. 4-(Fluoromethyl)phenyl phosphate acts as a mechanism-based inhibitor of calcineurin. *J. Biol. Chem.* **1995**, *270*, 25651−25655.
(53) Fruman, D. A.; Klee, C. B.; Bierer, B. E.; Burakoff, S. J. Calcineurin phosphatase activity in T lymphocytes is inhibited by FK506 and cyclosporin A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3686−3690.
(54) Gaymes, T. J.; Cebrat, M.; Siemion, I. Z.; Kay, J. E. Cyclolioeopeptide A (CLA) mediates its immunosuppressive activity through cyclophilin-dependent calcineurin inactivation. *FEBS Lett.* **1997**, *418*, 224−227.
(55) Tachibana, K.; Scheuer, P. J.; Tsukitani, Y.; Kikuchi, H.; Van Engen, D.; Clardy, J.; Gopichand, Y.; Schmitz. F. J. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. *J. Am. Chem. Soc.* **1981**, *103*, 2469−2471.
(56) Schmitz, F. J.; Prasad, R. S.; Gopichand, Y.; Hossain, M. B.; van der Helm, D.; Schmidt, P. Acanthifolicin, a new episulfide-containing polyether carboxylic acid from extracts of the marine sponge *Pandaros acanthifolium*. *J. Am. Chem. Soc.* **1981**, *103*, 2467−2469.
(57) Tsuboi, K.; Ichikawa, Y.; Isobe, M. Synthesis of okadaic acid-tautomycin hybrid. *Synlett* **1997**, 713−715.
(58) Cheng, X.-C.; Kihara, T.; Kusakabe, H.; Magae, J.; Kobayashi, Y.; Fang, R.-P.; Ni, Z.-F.; Shen, Y.-C.; Ko, K.; Yamaguchi, I.; Isono, K. A new antibiotic, Tautomycin. *J. Antibiot.* **1987**, *40*, 907−909.
(59) Cheng, X.-C.; Kihara, T.; Ying, X.; Uramoto, M.; Osada, H.; Kusakabe, H.; Wang, B.-N.; Kobayashi, Y.; Ko, K.; Yamaguchi, I.; Isono, K. A new antibiotic, Tautomycetin. *J. Antibiot.* **1989**, *42*, 141−144.
(60) Carmichael, W. W. Cyanobacteria secondary metabolites − the cyanotoxins. *J. Appl. Bacteriol.* **1992**, *72*, 445−459.
(61) Carmichael, W. W. The toxins of cyanobacteria. *Sci. Am.* **1994**, *270*, 78−86.
(62) Namikoshi, M. N.; Rinehart, K. L.; Sakai, R.; Stotts, R. R.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W.; Evans, W. R. Identification of 12 hepatotoxins from a Homer Lake bloom of the cynobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. *J. Org. Chem.* **1992**, *57*, 866−872.
(63) Dilip de Silva, E.; Williams, D. E.; Anderson, R. J.; Klix, H.; Holmes, C. F. B.; Allen, T. M. Motoporin, a potent protein phosphatase inhibitor isolated from the Papua New Guinea sponge *Theonella swinhoei Gray*. *Tetrahedron Lett.* **1992**, *33*, 1561−1564.

(64) Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K.; Fijita, S.; Furuya, T. Calyculin A. A novel antitumour metabolite from the marine sponge *Discodermia calyx*. *J. Am. Chem. Soc.* **1986**, *108*, 2780−2781.
(65) Kato, Y.; Fusetani, N.; Matsunaga, S.; Hashimoto, K. Bioactive marine metabolites. 24. Isolation and structure elucidation of calyculins B, C, and D, novel antitumor metabolites, from the marine sponge *Discodermia calyx*. *J. Org. Chem.* **1988**, *53*, 3930−3932.
(66) Ishihara, H.; Martin, B. L.; Brautigan, D. L.; Karaki, H.; Ozaki, H.; Kato, Y.; Fusetani, N.; Watabe, S.; Hashimoto, K.; Uemura, D.; Hartshorne, D. J. Calyculin A and okadaic acid: Inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun.* **1989**, *159*, 871−877.
(67) Volter, K. E.; Pierens, G. K.; Quinn, R. J.; Wakimoto, T.; Matsunaga, S.; Fusetani, N. The solution structures of calyculin A and dephosphonocalyculin A by NMR. *Bioorg. Med. Chem Lett.* **1999**, *9*, 717−722.
(68) Suzuki, T.; Suzuki, M.; Furusaki, A.; Matsumoto, T.; Kato, A.; Imanaka, Y.; Kurosawa, E. Constituents of marine plants. 62. Teurilene and thyrisiferyl 23-acetate, meso and remarkably cytotoxic compounds from the marine red alga *Laurencia obtus (Hudson) Lamouroux*. *Tetrahedron Lett.* **1985**, *26*, 1329−1332.
(69) Stampwala, S. S.; Bunge, R. H.; Hirley, T. R.; Wilmer, N. E.; Brankiewicz, A. J.; Steinman, C. E.; Smtka, T. A.; French, J. C. Novel antitumor agents CI-920, PD 113,270 and PD 113,271. II. Isolation and characterization. *J. Antibiot.* **1983**, *36*, 1601−1605.
(70) Southcott, C. V. Injuries from Coleoptera. *Med. J. Aust.* **1989**, *151*, 654−659.
(71) Gunasekera, S. P.; McCarthy, P. J.; Longley, R. E.; Pomponi, S. A.; Wright, A. E.; Lobkovsky, E.; Clardy, J. Discorhabdin P, a new enzyme inhibitor from a deep-water Carribean sponge of the genus *Batzella*. *J. Nat. Prod.* **1999**, *62*, 173−175.
(72) Egloff, M. P.; Cohen, P. T. W.; Reinemer, P.; Barford, D. Crystal structure of the catalytic subunit of human protein phosphatase 1 and its complex with tungstate. *J. Mol. Biol.* **1995**, *254*, 942−959.
(73) Goldberg, J.; Huang, H. B.; Kwon, Y. G.; Greengard, P.; Nairn, A. C.; Kuriyan, J. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. *Nature* **1995**, *376*, 745−753.
(74) Griffith, J. P.; Kim, J. L.; Kim, E. E.; Sintchak, M. D.; Thomason, J. A.; Fitzgibbon, M. J.; Fleming, M. A.; Caron, P. R.; Hsiao, K.; Navia, M. A. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12−FK506. *Cell* **1995**, *82*, 507−522.
(75) Kissinger, C. R.; Parge, H. E.; Knighton, D. R.; Lewis, C. T.; Pelletier, L. A.; Tempczyk, A.; Kalish, V. J.; Tucker, K. D.; Showalter, R. E.; Moomaw, E. W.; Gastinel, L. N.; Habuka, N.; Chen, X.; Maldonado, F.; Barker, J. E.; Villafranca, J. E. Crystal structures of human calcineurin and the human FKBP12−FK506 calcineurin complex. *Nature* **1995**, *378*, 641−644.
(76) Zhou, S.; Clemens, J. C.; Stone, R. L.; Dixon, J. E. Mutational analysis of a Ser/Thr phosphatase. Identificaiton of residues important in phosphoesterase substrate binding and catalysis. *J. Biol. Chem.* **1994**, *269*, 26234−26238.
(77) Huang, H. B.; Horiuchi, A.; Goldberg, J.; Greengard, P.; Nairn, A. C. Site-directed mutagensis of amino acid residies of protein phosphatase 1 involved in catalysis and inhibitor binding. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3530−3535.
(78) Zhang, J.; Zhang, Z.; Brew, K.; Lee, E. Y. C. Mutational analysis of the catalytic subunit of muscle protein phosphatase-1. *Biochemistry* **1996**, *35*, 6276−6282.
(79) Zhang, Z.; Zhao, S.; Long, F.; Zhang, L.; Bai, G.; Shima, H.; Nagao, M.; Lee, E. Y. A mutant of protein phosphatase-1 that exhibits altered toxin sensitivity. *J. Biol. Chem.* **1994**, *269*, 16997−17000.
(80) Shima, H.; Tohda, H.; Aonuma, S.; Nakayasu, M.; DePaoli-Roach, A. A.; Sugimura, T.; Nagao, M. Characterization of the PP2Aα gene mutation in okadaic acid-resistant variants of CHO−K1 cells. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9267−9271.
(81) Pinna, L. A.; Donella-Deana, A. Phosphorylated synthetic peptides as tools for studying protein phosphatases. *Biochim. Biophys. Acta* **1994**, *1222*, 415−431.
(82) Aldape, R. A.; Futer, O.; DeCenzo, M. T.; Jarrett, B. P.; Murcko, M. A.; Livingston, D. J. Charged surface residues of FKBP12 participate in formation of the FKBP12−FK506-calcineurin complex. *J. Biol. Chem.* **1992**, *267*, 16029−16032.
(83) Yang, D.; Rosen, M. K.; Schreiber, S. L. A composite FKB12-FK506 surface that contacts calcineurin. *J. Am. Chem. Soc.* **1993**, *115*, 819−820.
(84) Futer, O.; DeCenzo, M. T.; Aldape, R. A.; Livingston, D. J. FK506 binding protein mutational analysis. *J. Biol. Chem.* **1995**, *270*, 18935−18940.
(85) Krebs, B.; Ahlers, F.; Bremer, B.; Eulering, B.; Klabunde, T.; Schepers, K.; Schmidt, M.; Strater, N.; Than, R.; Witzel, H. Crystal structure of a purple acid phosphatase. Structural and functional studies on its model compounds. *Bioinorg. Chem.* **1997**, 412−425.
(86) Lanaras, T.; Cook, C. M.; Eriksson, J. E.; Meriluoto, T. A. O.; Hotokka, M. Computer modeling of the 3-dimensional structures of the cyanobacterial hepatotoxins microcystin-LR and nodularin. *Toxicon* **1991**, *29*, 901−906.
(87) Taylor, C.; Quinn, R. J.; McCulloch, R.; Nishiwaki-Matsushima, R.; Fujiki, H. An alternative computer model of the 3-dimensional structures of microcystin-LR and nodularin rationalising their interactions with protein phosphatases 1 and 2A. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 299−302.
(88) Bagu, J. R.; Sykes, B. D.; Craig, M. M.; Holmes, C. F. B. A molecular basis for different interactions of marine toxins with protein phosphatase-1. Molecular models for bound motuporin, microcystins, okadaic acid, and calyculin A. *J. Biol. Chem.* **1997**, *272*, 5087−5097.
(89) Gupta, V.; Ogawa, A. K.; Du, X.; Houk, K. N.; Armstrong, R. W. A model for binding of structurally diverse natural product inhibitors of protein phosphatases PP1 and PP2A. *J. Med. Chem.* **1997**, *40*, 3199−3206.
(90) Gauss, C. M.; Sheppeck, J. E.; Nairn, A. C.; Chamberlin, A. R. A molecular modeling analysis of the binding interactions between the okadaic acid class of natural product inhibitors and the Ser-Thr phosphatases, PP1 and PP2A. *Bioorg. Med. Chem.* **1997**, *5*, 1751−1773.
(91) Aggen, J. B.; Humphrey, J. M.; Gauss, C.-M.; Huang, H.-B.; Nairn, A. C.; Chamberlin, A. R. The design, synthesis, and biological evaluation of analogues of the serine-threonine protein phosphatase 1 and 2A inhibitor microcystin-LA: Rational modifications imparting PP1 selectivity. *Bioorg. Med. Chem.* **1999**, *7*, 543−564.
(92) Hu, T.; Curtis, J. M.; Walter, J. A.; Wright, J. L. C. Identification of DTX-4, a new water-soluble phosphatase inhibitor from the toxic dinoflagellate *Prorocentrum lima*. *J. Chem. Soc., Chem. Commun.* **1995**, 597−598.
(93) Nishiwaki, S.; Fujiki, H.; Suganuma, M.; Furuya-Suguri, H.; Matsushima, R.; Iida, Y.; Ojika, M.; Yamada, K.; Uemura, D.; Yasumoto, T.; Schmitz, F. J.; Sugimura, T. Structure activity relationship within a series of okadaic acid derivatives. *Carcinogenesis* **1990**, *11*, 1837−1841.
(94) Takai, A.; Murata, M.; Torigoe, K.; Isobe, M.; Mieskes, G.; Yasumoto, T. Inhibitory effect of okadaic acid derivatives on protein phosphatases. A study on structure-affinity relationship. *Biochem. J.* **1992**, *284*, 539−544.
(95) Saskai, K.; Murata, M.; Yasumoto, T.; Mieskes, G.; Takai, A. Affinity of okadaic acid to type-1 and type-2A protein phosphatases is markedly reduced by oxidation of its 27-hydroxyl group. *Biochem. J.* **1994**, *298*, 259−262.
(96) Frydrychowski, V. A.; Urbanek, R. A.; Dounay, A. B.; Forsyth, C. J. The importance of the C28−C38 hydrophobic domain of Okadaic Acid for potent inhibition of protein Serine-Threonine phosphatases 1 and 2A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 647−649.
(97) Sugiyama, Y.; Ohtani, I. I.; Isobe, M.; Takai, A.; Ubukata, M.; Isono, K. Molecular shape analysis and activity of tautomycin, a protein phosphatase inhibitor. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 3−8.
(98) Takai, A.; Tsuboi, K.; Koyasu, M.; Isobe, M. Effects of modification of the hydrophobic C1−C16 segment of tautomycin on its affinity to type-1 and type-2A protein phosphatses. *Biochem. J.* **2000**, *350*, 81−88.
(99) Namikoshi, M. N.; Sun, F. R.; Choi, B. W.; Rinehart, K. L.; Carmichael, W. W.; Evans, W. R. Seven more microcystins from Homer Lake cells: Application of the general method for structure assignment of peptides containing α,β-dehydroamino acid unit(s). *J. Org. Chem.* **1995**, *60*, 3671−3679.
(100) Stotts, R. R.; Namikoshi, M. H.; Hascheck, W. M.; Rinehart, K. L.; Carmichael, W. W.; M., D. A.; Beasley, V. R. Structural modifications imparting reduced toxicity in microcyctins from *Microcystis Spp. Toxicon* **1993**, *31*, 783−789.
(101) Harada, K.-I.; Matsuura, K.; Suzuki, M.; Watanabe, M. F.; Oishi, S.; Dahlem, A. M.; Beasley, V. R.; Carmichael, W. W. Isolation and characterisation of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). *Toxicon* **1990**, *28*, 55−64.
(102) Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Sivonen, K.; Carmichael, W. W. Structures of three new cyclic heptapeptide hepatotoxins produced by the cyanobacterium (blue-green alga) *Nostoc* sp. strain 152. *J. Org. Chem.* **1990**, *55*, 6135−6139.
(103) Sivonen, K.; Carmichael, W. W.; Namikoshi, M.; Rinehart, K. L.; Dalhem, A. M.; Niemela, S. I. Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium *Nostoc* sp. strain 152. *Appl. Environ. Microbiol.* **1990**, *56*, 2650−2657.

(104) Sivonen, K.; Namikoshi, M.; Evans, W. R.; Fardig, M.; Carmichael, W. W.; Rinehart, K. L. Three new microcystins, cyclic heptapeptide hepatotoxins, from *Nostoc* sp. strain 152. *Chem. Res. Toxicol.* **1992**, *5*, 464−469.
(105) Namikoshi, M. N.; Rinehart, K. L.; Sakai, R.; Sivonen, K.; Carmichael, W. W. Identification of 12 hepatotoxins from a Homer Lake bloom of the cyanobacteria Microcystis aeruginosa, Microcystis viridis, and Microcystis wesenbergii: nine new microcystins *J. Org. Chem.* **1992**, *57*, 866−872.
(106) Choi, B. W.; Namikohshi, M.; Sun, F. R.; Rinehart, K. L.; Carmichael, W. W.; Kaup, A. M.; Evans, W. R.; Beasley, V. Isolation of linear peptides related to the hepatotoxins nodularin and microcystins. *Tetrahedron Lett.* **1993**, *34*, 7881−7884.
(107) Kishikawa, K.; Chalfant, C. E.; Perry, D. E.; Bielawska, A.; Hannun, Y. A. Phosphatidic acid is a potent and selctive inhibitor of protein phosphatase 1 and an inhibitor of Ceramide-mediated response. *J. Biol. Chem.* **1999**, *274*, 21335−21341.
(108) Matsunaga, S.; Fujiki, H.; Sakata, D. Calyculins E, F, G, and H, additional inhibitors of protein phosphatases 1 and 2A, from the marine sponge *Discodermia calyx*. *Tetrahedron* **1991**, *47*, 2999−3006.
(109) Fujiki, H.; Suganuma, M. Tumour promotion by inhibitors of protein phosphatases 1 and 2A: The okadaic acid class of compounds. *Adv. Cancer Res.* **1993**, *53*, 143−194.
(110) Wipf, P.; Cunningham, A.; Rice, R. L.; Lazo, J. S. Combinatorial synthesis and biological evaluation of library of small-molecule Ser/Thr-protein phosphatase inhibitors. *Bioorg. Med. Chem.* **1997**, *5*, 165−177.
(111) Tatlock, J. H.; Linton, M. A.; Hou, X. J.; Kissinger, C. R.; Pelletier, L. A.; Showalter, R. E.; Tempczyk, A.; Villafranca, J. E. Structure-based design of novel calcineurin (PP2B) inhibitors. *Bioorg. Med. Chem Lett.* **1997**, *8*, 1007−1012.
(112) Sodeoka, M.; Baba, Y.; Kobayashi, S.; Hirukawa, N. Structure−activity relationships of cantharidin derivatives to protein phosphatases 1, 2A (1), and 2B. *Bioorg. Med. Chem.* **1997**, *7*, 1833−1836.
(113) Enz, A.; Zenke, G.; Pombo-Villar, E. 7-oxa[2.2.1] bicycloheptane-2,3-dicarboxylic acid derivatives as phosphatase inhibitors. *Bioorg. Med. Chem.* **1997**, *7*, 2513−2518.
(114) McCluskey, A.; Bowyer, M. C.; Collins, E.; Sim, A. T. R.; Sakoff, J. A.; Baldwin, M. L. Anhydride modified cantharidin analogues: Synthesis, anti-cancer activity and selective inhibition of protein phosphatase 1 and 2A. *Bioorg. Med. Chem Lett.* **2000**, *10*, 1687−1690.
(115) McCluskey, A.; Keane, M. A.; Mudgee, L.-M.; Sim, A. T. R.; Sakoff, J.; Quinn, R. J. Anhydride modified Cantharidin analogues. Is ring opening important in the inhibition of protein phosphatase 2A. *Eur. J. Med. Chem.* **2000**, *35*, 957−964.
(116) McCluskey, A.; Taylor, C.; Quinn, R. J.; Suganuma, M.; Fujiki, H. Inhibition of protein phosphatase 2A by Cantharidin analogues *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1025−1028.
(117) McCluskey, A.; Bowyer, M. C.; Walkom, C.; Ackland, S. P.; Gardiner, E.; Sakoff, J. A. Cantharimides: A new class of modified cantharidin analogues inhibiting protein phosphatases 1 and 2A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2941−2946.
(118) Laidley, C. W.; Dauben, W. G.; Guo, Z. R.; Lam, J. Y. L.; Casida, J. E. 2-carboxymethylendothal analogues as affinity probes for stabilized protein phosphatase 2A. *Bioorg. Med. Chem.* **1999**, *7*, 2937−2944.
(119) Ho, S.; Clipstone, N.; Timmermann, L.; Northrop, J.; Graef, I.; Fiorentino, D.; Nourse, J.; Crabtree, G. R. The mechanism of action of cyclosporin A and FK506. *Clin. Immunol. Immunopathol.* **1996**, *80*, S40−S45.
(120) Yan, M. S.; Xiue, S.; Wei, L. X.; Piao, X. H. The preliminary observation on immunosuppressive effect of norcantharidin in mice. *Immunopharmacol. Immunotoxicol.* **1993**, *15*, 79−85.
(121) Clementi, G.; Amico-Roxas, M.; Caruso, A.; Catena-Cutuli, V. M.; Prato, A.; Maugeri, S.; de Bernadis, E.; Scapagnini, U. Effects of CGRP in different models of mouse ear inflammation. *Life Sci.* **1994**, *54*, 119−124.
(122) McCluskey, A.; Keane, M. A.; Walkom, C.; Bowyer, M. C.; Sim, A. T. R.; Young, D. J.; Sakoff, J. A. The first two cantharidin analogues displaying PP1 selectivity. *Bioorg. Med. Chem Lett.* In press.
(123) Isobe, M.; Ichikawa, Y.; Goto, T. Synthetic studies toward marine toxic polyethers. 5. The total synthesis of okadaic acid. *Tetrahedron Lett.* **1986**, 27, 963−966.
(124) Isobe, M.; Ichikawa, Y.; Bai, D.; Masaki, H.; Goto, T. Synthesis of a marine polyether toxin, okadaic acid. IV. Total synthesis. *Tetrahedron* **1987**, *43*, 4767−4776.
(125) Forsyth, C. J.; Sabes, S. F.; Urbanek, R. A. An efficient total synthesis of Okadaic Acid. *J. Am. Chem. Soc.* **1997**, *119*, 8381−8382.
(126) Dounay, A. B.; Urbanek, R. A.; Frydrychowski, V. A.; Forsyth, C. J. Expedient access to the Okadaic Acid architecture: A novel synthesis of the C1−C27 domain. *J. Org. Chem.* **2001**, *66*, 925−938.
(127) Urbanek, R. A.; Sabes, S. F.; Forsyth, C. J. Efficient synthesis of Okadaic Acid. 1. Convergent assembly of the C15−C38 domain. *J. Am. Chem. Soc.* **1998**, *120*, 2523−2533.
(128) Sabes, S. F.; Urbanek, R. A.; Forsyth, C. J. Efficient synthesis of Okadaic Acid. 2. Synthesis of the C1−C14 domain and the completion of the total synthesis. *J. Am. Chem. Soc.* **1998**, *120*, 2534−542.
(129) Ley, S. V.; Humphries, A. C.; Eick, H.; Downham, R.; Ross, A. R.; Boyce, R. J.; Pavey, J. B.; Pietruszka, J. Total synthesis of the protein phosphatase inhibitor Okadaic acid. *J. Chem. Soc., Perkin Trans. 1* **1998**, 3907−3912.
(130) Marko, I. E.; Dobbs, A. P.; Scheirmann, V.; Chelle, F.; Bayston, D. J. Concise and stereocontrolled assembly of substituted dihydropyrans. Synthetic studies towards the trans-dioxadecalin subunit of Okadaic Acid. *Tetrahedron Lett.* **1997**, *38*, 2899−2902.
(131) Marko, I. E.; Chelle, F. Efficient methodology for the construction of substituted spiroketals. Model studies towards the synthesis of the eastern spiroketal subunit of Okadaic Acid. *Tetrahedron Lett.* **1997**, *38*, 2895−2898.
(132) Dankwardt, S. M.; Dankwardt, J. W.; Schlessinger, R. H. A vinylogous urethane approach towards the synthesis of Okadaic Acid. Construction of the C1−C8 fragment. Part I. *Tetrahedron Lett.* **1998**, *39*, 4971−4974.
(133) Dankwardt, S. M.; Dankwardt, J. W.; Schlessinger, R. H. A vinylogous urethane approach towards the synthesis of Okadaic Acid. Construction of the C9−C18 fragment. Part II. *Tetrahedron Lett.* **1998**, *39*, 4975−4978.
(134) Dankwardt, J. W.; Dankwardt, S. M.; Schlessinger, R. H. Synthesis of the C19 through C27 segment of Okadaic Acid using vinylogous urethane Aldol chemistry: Part III. *Tetrahedron Lett.* **1998**, *39*, 4979−4982.
(135) Dankwardt, S. M.; Dankwardt, J.; Schlessinger, R. H. Synthesis of the C28 through C38 segment of Okadaic Acid using vinylogous urethane Aldol chemistry: Part IV. *Tetrahedron Lett.* **1998**, *39*, 4983−4986.
(136) Dounay, A. B.; Forsyth, C. J. Abbreviated synthesis if the C3−C14 (substituted 1,7-dioxaspiro[5.5]undec-3-ene) system of okadaic acid. *Org. Lett.* **1999**, *1*, 451−453.
(137) Dounay, A. B.; Urbanek, R. A.; Sabes, S. F.; Forsyth, C. J. Total synthesis of the marine natural product7-deoxy-okadaic acid. A potent inhibitor of serine/threonine-specific protein phosphatases. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 2258−2262.
(138) Maurer, K. W.; Armstrong, R. W. Synthesis if the C1−C21 fragment of the serine/threonine phosphatase inhibitor Tautomycin. *J. Org. Chem.* **1996**, *61*, 3106−3116.
(139) Oikawa, M.; Oikawa, H.; Ichihara, A. Synthetic study on tautomycin − stereocontrolled synthesis if C_1−C_{18} fragment using a strategy of seelctive reduction of spiroketal. *Tetrahedron Lett.* **1993**, *30*, 4797−4800.
(140) Oikawa, H.; Oikawa, M.; Ueno, T.; Ichihara, A. Total synthesis of tautomycin − efficient aldol coupling of two latge subunits. *Tetrahedron Lett.* **1994**, *35*, 4809−4812.
(141) Oikawa, M.; Ueno, T.; Oikawa, H.; Ichihara, A. Total synthesis of tautomycin. *J. Org. Chem.* **1995**, *60*, 5048−5068.
(142) Ueno, T.; Oikawa, M.; Oikawa, H.; Ichihara, A. Synthestic study oftautomycin and tautomycetin − stereocontrolled construction of the dialkylmaleic anhydride segment. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 2104−2110.
(143) Ichikawa, Y.; Naganawa, A.; Isobe, M. Synthesis of the 2,3-disubstituted maleic anhydride segment of tautomycin. *Synlett* **1993**, 737−738.
(144) Ichikawa, Y.; Tsuboi, K.; Naganawa, A.; Isobe, M. Synthesis of the (+)-2,3-disubstituted maleic anhydfride segment and (+)-enone segment with C_{25} through C_{19} of tautomycin. *Synlett* **1993**, 907−908.
(145) Naganawa, A.; Ichikawa, Y.; Isobe, M. Synthetic studies on tautomycin synthesis of 2,3-disubstituted maleic anhydride segment. *Tetrahedron* **1994**, *50*, 8969−8982.
(146) Jiang, Y. M.; Ichikawa, Y.; Isobe, M. Synthesis of segment C of tautomycin. *Synlett* **1995**, *3*, 285−288.
(147) Ichikawa, Y.; Tsuboi, K.; Jaing, Y.; Naganawa, A.; Isobe, M. Total synthesis of (+)-tautomycin. *Tetrahedron Lett.* **1995**, *36*, 7101−7104.
(148) Jaing, Y.; Isobe, M. Pseudoenantiomeric heteroconjugate addition approach for synthesis of spiro segment of tautomycin. *Tetrahedron* **1996**, *52*, 2877−2892.
(149) Nakamura, S.; Shibasaki, M. Synthetic studies on tautomycin − stereoselective construction of the C_1−C_{26} region. *Tetrahedron Lett.* **1994**, *35*, 4145−4148.
(150) Sheppeck, J. E., II; Liu, W.; Chamberlin, A. R. Total synthesis of the serine/threonine-specific protein phosphatase inhibitor tautomycin. *J. Org. Chem.* **1997**, *62*, 387−398.
(151) Marshall, J. A.; Yanik, M. M. Synthesis of a C1−C21 subunit of the protein phosphaatse inhibitor tautomycin: A formal total synthesis. *J. Org. Chem.* **2001**, *66*, 1373−1379.
(152) Humphrey, J. M.; Aggen, J. B.; Chamberlin, A. R. Total synthesis of the serine-threonine phospatase inhibitor Microcystin-LA. *J. Am. Chem. Soc.* **1996**, *118*, 11759−11770.

(153) Mehrotra, A. P.; Webbster, K. L.; Gani, D. Design and prepartation of serine-threonine protein phosphatase inhibitors based upon the nodularin and microcycstin toxin structures. 1. Evaluation of key features and synthesis of a rationally stripped-down nodularin macrocycle. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2495−2511.

(154) Maude, A. B.; Mehrotra, A. M.; Gani, D. Design and preparation of serine-threonine protein phosphatase inhibitors based upon the nodularin and microcystin toxin structures. Part 2. Synthesis of a functionalised nodularin macrocycle and a stripped-down microcystin macrocysle. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2513−2526.

(155) Webster, K. L.; Maude., A. B.; O'Donnell, M. E.; Mehrotra, A. P.; Gani, D. Design and preparation of serine-threonine protein phosphatase inhibitors based upon the nodularin and microcystin toxin structures. Part 3. *J. Chem. Soc., Perkin Trans. 1* **2001**, 1673−1695.

(156) Mehrotra, A. P.; Gani, D. Synthesis of functionalised cyclic pentapeptide analogues of the serine-threonine protein phosphatase inhibitor nodularin. *Tetrahedron Lett.* **1996**, *37*, 6915−6918.

(157) Samy, R.; Kim, H. Y.; Brady, M.; Toogood, P. L. Total synthesis of Motuporin and 5-[L-Ala]-Motuporin. *J. Org. Chem.* **1999**, *64*, 2711−2728.

(158) Kim, H. Y.; Stein, K.; Toogood, P. L. Synthesis towards microcystins and related toxins. *J. Chem. Soc., Chem. Commun.* **1996**, 1683−1684.

(159) Chakraborty, T. K.; Joshi, S. P. Total synthesis of N-phthaloyl Adda methyl ester: all stereocentres originating from a single chiral epoxy alcohol. *Tetrahedron Lett.* **1990**, *31*, 2043−2046.

(160) Beatty, M. F.; Jennings-White, C.; Avery, M. A. Stereocontrolled synthesis of (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), the amino acid chraracteristic of microcystins and nodularin. *J. Chem. Soc., Perkin Trans. 1*, **1992**, 1637−1641.

(161) Beatty, M. F.; Jennings-White, C.; Avery, M. A. Stereocontrolled synthesis of (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), the amino acid chraracteristic of microcystins and nodularin. *J. Chem. Soc., Chem. Commun.* **1991**, 351−352.

(162) D'aniello, F.; Mann, A.; Taddei, M. 1,3-Stereocontrol with bromoallenes. Synthesis of *N*-Boc-Adda, the unique amino acid present in several inhibitors of serine/threonine phosphatases. *J. Org. Chem.* **1996**, *61*, 4870−4871.

(163) Sin, N.; Kallmerten, J. Synthesis of (2*S*,3*S*,8*S*,9*S*)-ADDA from glucose. *Tetrahedron Lett.* **1996**, *37*, 5645−5648.

(164) Panek, J.; Hu, T. Asymmetric synthesis of (2*S*,3*S*,8*S*,9*S*)-N−Boc-ADDA: Application of a Palladium(0)-catalysed cross-coupling reaction of trisubstututed olefins. *J. Org. Chem.* **1997**, *62*, 4914−4915.

(165) Cundy, D. J.; Donohue, A. C.; McCarthy, T. D. Towards an asymmetric synthesis of ADDA conjugates. *Tetrahedron Lett.* **1998**, *39*, 5125−5128.

(166) O'Donnell, M. E.; Sanvoisin, J.; Gani, D. Serine-threonine protein phosphatase inhibitors derived from nodularin: role of the 2-methyl and 3-diene groups in the Adda residue and the effect of macrocyclic conformational restraint. *J. Chem. Soc., Perkin Trans. 1* **2001**, 1696−1708.

(167) Webster, K. L.; Rutherford, T. J.; Gani, D. Comparison of solution-phase and solid-phase synthesis of a restrained proline-containing analogue of the Nodularin macrocycle. *Tetrahedron Lett.* **1997**, *38*, 5713−5716.

(168) Valentekovich, R. J.; Schreiber, S. L. Enantiospecific total synthesis of the protein phosphatase inhibitor Motuporin. *J. Am. Chem. Soc.* **1995**, *117*, 9069−9070.

(169) Hu, T.; Panek, J. S. Total synthesis of (−)-Motuporin. *J. Org. Chem.* **1999**, *64*, 3000−3001.

(170) Bauer, S. M.; Armstrong, R. W. Total synthesis of Motuoprin (Nodularin-V). *J. Am. Chem. Soc.* **1999**, *121*, 6355−6366.

(171) Ogawa, A. K.; Armstrong, R. W. Total synthesis of Calyculin C. *J. Am. Chem. Soc.* **1998**, *120*, 12435−12442.

(172) Matsunaga, S.; Wakimoto, T.; Fusetani, N. Isolation of four new Calyculins from the marine sponge *Discodermia* calx. *J. Org. Chem.* **1997**, *62*, 2640−2642.

(173) Matsunaga, S.; Wakimoto, T.; Fusetani, N.; Suganuma, M. Isolation of dephosphonocalyculin A from the marine sponge *Discodermia calyx*. *Tetrahedron Lett.* **1997**, *38*, 3763−3764.

(174) Ogawa, A. K.; DeMattei, J. A.; Scarlato, G. R.; Tellew, J. E.; Chong, L. S.; Armstrong, R. W. Asymmetric synthesis of Calyculin C. 2. Synthesis of the $C_{26}-C_{37}$ fragment and model Wittig couplings. *J. Org. Chem.* **1996**, *61*, 6153−6161.

(175) Scarlato, G. R.; DeMattei, J. A.; Chong, L. S.; Ogawa, A. K.; Lin, M. R.; Armstrong, R. W. Asymmetric synthesis of Calyculin C. 1. Synthesis of the C_1-C_{25} fragment. *J. Org. Chem.* **1996**, *61*, 6139−6152.

(176) Zhao, Z.; Scarlato, G. R.; Armstrong, R. W. Synthesis of *trans*-4-alkenyl oxazoles. *Tetrahedron Lett.* **1991**, *32*, 1609−1612.

(177) Armstrong, R. W.; DeMattei, J. A. Stereoselective methylations of bicyclic lactams derived from pyroglutamic acid. *Tetrahedron Lett.* **1991**, *32*, 5749−5452.

(178) Evans, D. A.; Gage, J. R.; Leighton, J. L. Total synthesis of (+)-calyculin A. *J. Am. Chem. Soc.* **1992**, *114*, 9434−9453.

(179) Tanimoto, N.; Gerritz, S. W.; Sawabe, A.; Noda, T.; Filla, S. A.; Masamume, S. The synthesis of naturally occurring (−)-Calyculin A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 673−765.

(180) Yokokawa, F.; Hamada, Y.; Shioiri, T. Total synthesis of Calyculin A − Construction of the C_9-C_{37} fragment. *J. Chem. Soc., Chem. Commun.* **1996**, 871−872.

(181) Pihko, P. M.; Koskinen, A. M. P. Synthesis of the C26−C32 oxazole fragment of Calyculin C: A test case for oxazole synthesis. *J. Org. Chem.* **1998**, *63*, 92.

(182) Barrett, A. G. M.; Malecha, J. W. Synthetic studies on Calyculin A − A convenient asymmetric synthesis of anti-vicinal diols. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1901−1905.

(183) Evans, D. A.; Gage, J. R.; Leighton, J. L. Asymmetric synthesis of calyculin A. 3. Assemblage of the calyculin skeleton and the introduction of a new phosphate monoester synthesis. *J. Org. Chem.* **1992**, *57*, 1964−1966.

(184) Evans, D. A.; Gage, J. R.; Leighton, J. L.; Kim, A. S. Asymmetric synthesis of calyculin A. 2. The $C_{26}-C_{37}$ γ-amino acid fragments. *J. Org. Chem.* **1992**, *57*, 1961−1963.

(185) Evans, D. A.; Gage, J. R. Asymmetric synthesis of calyculin A. 1. The C_1-C_{25} spiroketal fragment. *J. Org. Chem.* **1992**, *57*, 1958−1961.

(186) Evans, D. A.; Gage, J. R. Reversal of aldehyde diastereofacial selectivity in a methyl ketonealdol reaction. Application to the synthesis of the calyculin spiroketal. *Tetrahedron Lett.* **1990**, *31*, 6129−6132.

(187) Vaccaro, H. A.; Levy, D. E.; Sawabe, A.; Jaetsch, T.; Masamume, S. Synthesis of calyculin: a synthetic intermediate corresponding to the C(26)−C(37) fragment. *Tetrahedron Lett.* **1992**, *33*, 1937−1940.

(188) Duplantier, A. J.; Nantz, M. H.; Roberts, J. C.; Short, R. P.; Somfai, P.; Masamume, S. Triple asymmetric synthesis of fragment assembly. Validity of approximate multiplicity of the three diastereofacial selectivities. *Tetrahedron Lett.* **1989**, *30*, 7357−7360.

(189) Kabeya, M.; Hamada, Y.; Shioiri, T. An alternative efficient synthesis of the C20−C25 building unit for Calyculin A. *Tetrahedron* **1997**, *53*, 9777−9788.

(190) Kabeya, M.; Hamada, Y.; Shioiri, T. Synthesis of γ-lactone derivatives as key intermediates for the spiroketal fragment in Calyculin A. *Tetrahedron* **1997**, *53*, 9769−9776.

(191) Hamada, Y.; Yokokawa, F.; Kabeya, M.; Hatano, K.; Kurono, Y.; Shioiri, T. An efficient synthesis of C20−C25 building blocks for Calyculin A. *Tetrahedron* **1996**, *52*, 8297−8306.

(192) Yokokawa, F.; Hamada, Y.; Shioiri, T. Synthesis of the tetraene fragment of Calycilins having natural configurations. *Tetrahedron Lett.* **1993**, *34*, 6559−6562.

(193) Yokokawa, F.; Hamada, Y.; Shioiri, T. A synthesis of the oxazole part of calyculins. Part 1. *Synlett* **1992**, 149−150.

(194) Yokokawa, F.; Hamada, Y.; Shioiri, T. A synthesis of the oxazole part of calyculins. Part 2. *Synlett* **1992**, 151−152.

(195) Yokokawa, F.; Hamada, Y.; Shioiri, T. Synthesis of 2,4-disubstituted oxazoles. *Synlett* **1992**, 153−155.

(196) Yokokawa, F.; Hamada, Y.; Shioiri, T. Synthesis of the C26−C37 fragment of calyculin A having natural configuration. *Synlett* **1992**, 703−705.

(197) Yokokawa, F.; Hamada, Y.; Shioiri, T. Synthesis of the tetraene fragment of calyculins having natural configurations. *Tetrahedron Lett.* **1992**, *34*, 6559−6562.

(198) Yokokawa, F.; Hamada, Y.; Shioiri, T. Total synthesis of calyculinA − construction of the C(9)−C(37) fragment. *J. Chem. Soc., Chem. Commun.* **1996**, 871−872.

(199) Takebuchi, K.; Hamada, Y.; Shioiri, T. Synthesis of the C13−C19 unit in the spiroketal fragment of calyculins. *Tetrahedron Lett.* **1994**, *35*, 5239−5242.

(200) Hara, O.; Hamada, Y.; Shiori, T. New methods and reagents in organic synthesis. 93. Stereoselective synthesis of the C13−C19 fragment of the cytotoxic marine products, calyculins. Part 1. *Synlett* **1991**, 283−284.

(201) Hara, O.; Hamada, Y.; Shioiri, T. New methods and reagents in organic synthesis. 94. Stereoselective synthesis of the C13−C19 fragment of the cytotoxic marine products, calyculins. Part 2. *Synlett* **1991**, 285−286.

(202) Smith, A. B., III.; Iwashima, M. Calyculin synthetic studies. 4. Remarkable reversal of diastereoselectivity in Payne epoxidation of vinyl spiroketal intermediates. *Tetrahedron Lett.* **1994**, *35*, 6051−6052.

(203) Salvatore, B. A.; Smith, A. B., III. Calyculin synthetic studies. 3. Enantiomeric purity determination for the C26−C32 oxazole segment via the Silks-Odom Se77 NMR method. *Tetrahedron Lett.* **1994**, *35*, 1329−1330.

(204) Smith, A. B., III.; Salvatore, B. A.; Hull, K. G.; Duan, J. J.-W. Calyculin synthetic studies. 2. Stereocontrolled assembly of the C(9)−C(13) dithiane and C(26)−C(37) oxazole intermediates. *Tetrahedron Lett.* **1991**, *32*, 4859−4862.
(205) Smith, A. B., III.; Duan, J. J.-W.; Hull, K. G.; Salvatore, B. A. Calyculin synthetic studies. Stereoselective construction of the C(14)−C(25) spiroketal subunit. *Tetrahedron Lett.* **1991**, *32*, 4855−4858.
(206) Barrett, A. G. M.; Edmunds, J. J.; Hendrix, J. A.; Malecha, J. W.; Parkinson, C. J. Stereocontrolled synthesis of calyculin A: construction of the C(26)−C(37) amide-oxazole unit. *J. Chem. Soc., Chem. Commun.* **1992**, 1240−1242.
(207) Barrett, A. G. M.; Edmunds, J. J.; Hendrix, J. A.; Horita, K.; Parkinson, C. J. Stereocontrolled synthesis of calyculin A: construction of the C(1)−C(14) tetraene nitrile unit. *J. Chem. Soc., Chem. Commun.* **1992**, 1238−1240.
(208) Barrett, A. G. M.; Edmunds, J. J.; hendrix, J. A.; Horita, K.; Parkinson, C. J. Stereocontrolled synthesis of calyculin A: construction of the C(15)−C(25) spiro ketal unit. *J. Chem. Soc., Chem. Commun.* **1992**, 1236−1238.
(209) Barrett, A. G. M.; Malecha, J. W. B-[3-((Diisopropylamino)-dimethylsilyl)allyl]diisopinocamphenylborane: an excellent reagent for the stereoselective synthesis of anti-vicinal diols. *J. Org. Chem.* **1991**, *56*, 5243−5245.
(210) Koskinen, A. M. P.; Pihko, P. M. Calyculins − asymmetric synthesis of the C26−C32 fragment. *Tetrahedron Lett.* **1994**, *35*, 7417−7420.
(211) Koskinen, A. M. P.; Chen, J. Enantiospecific synthesis of polyhydroxyamino acids. Synthesis of the C33−C38 portion of calyculins. *Tetrahedron Lett.* **1991**, *32*, 6977−6980.
(212) Trost, B. M.; Flygare, J. A. A synthesis of the spiroketal subunit of (−)-Calyculin A. *Tetrahedron Lett.* **1994**, *35*, 4059−4062.
(213) Smith, A. B., III.; Friestad, G. K.; Duan, J. J.-W.; Barbosa, J.; Hull, K. G.; Iwashima, M.; Qiu, Y.; Spoors, P. G.;. Bertounesque, B.; Salvatore, B. A. Total synthesis of (+)-Calyculin A and (−)-Calyculin B. *J. Org. Chem.* **1998**, *63*, 7596−7597.
(214) Smith, A. B., III.; Friestad, G. K.; Barbosa, J.; Bertounesque, E.; Hull, K. G.; Iwashima, M.; Qiu, Y.; Salvatore, B. A.; Spoors, P. G.; Duan, J. J.-W. Total synthesis of (+)-Calyculin A and (−)-Calyculin B: Asymmetric synthesis of the C(9−25) spiroketal dipropionate subunit. *J. Am. Chem. Soc.* **1999**, *121*, 10468−10477.
(215) Smith, A. B., III.; Friestad, G. K.; Barbosa, J.; Bertounesque, E.; Duan, J. J.-W.; Hull, K. G.; Iwashima, M.; Qiu, Y.; Spoors, P. G.; Salvatore, B. A. Total synthesis of (+)-Calyculin A and (−)-Calyculin B: Cyanotetraene construction, asymmetric synthesis of the C(26−37) oxazole, fragment assembly, and final elaboration. *J. Am. Chem. Soc.* **1999**, *121*, 10478−10486.
(216) Gonzalez, I. C.; Forsyth, C. J. Total synthesis of thyrsiferyl 23-acetate, a specific inhibitor of protein phosphatase 2A and an anti-leukemic inducer of apoptosis. *J. Am. Chem. Soc.* **2000**, *122*, 9099−9108.
(217) Hashimoto, M.; Kan, T.; Nozaki, K.; Yanagiya, M.; Shirahama, H.; Matsumoto, T. Total syntheses of (+)-thyrsiferol, (+)-thyrsiferyl 23-acetate, and (+)-venustatriol. *J. Org. Chem.* **1990**, *55*, 5088−5107.
(218) Boger, D. L.; Ichikawa, S.; Zhong, W. Total synthesis of fostriecin (CI-920) *J. Am. Chem. Soc.* **2001**, *123*, 4161−4167.
(219) Just, G.; O'Connor, B. Synthesis of the 5R, 8R, 9S, 11R dephosphorylated derivative of CI-920, a novel antitumor agent. *Tetrahedron Lett.* **1988**, *29*, 753−756.
(220) Dauben, W. G.; Lam, J. Y. L.; Guo, Z. R. Total synthesis of (−)-Palasonin and (+)-Palasonin and related chemistry. *J. Org. Chem.* **1996**, *61*, 4816−4819.
(221) Kawamura, N.; Li, Y.; Engel, J. L.; Dauben, W. G.; Casida, J. E. Endothall thioanhydride: structural aspects of unusually high mouse toxicity and specific binding site in liver. *Chem. Res. Toxicol.* **1990**, *3*, 318−324.
(222) Rydberg, D. B.; Meinwald, J. Synthesis of (±)-Palasonin. *Tetrahedron Lett.* **1996**, *37*, 1129−1132.
(223) Dauben, W. G.; Kessel, C. R.; Takemura, K. H. Simple, efficient total synthesis of cantharidin via a high-pressure Diels−Alder reaction. *J. Am. Chem. Soc.* **1980**, *102*, 6893−6894.
(224) Dauben, W. G.; Gerdes, J. M.; Smith, D. B. Organic reactions at high pressure. The preparative scale synthesis of cantharidin. *J. Org. Chem.* **1985**, *50*, 2576−2578.
(225) Price, N. E.; Mumby, M. C. Brain protein serine/threonine phosphatases. *Curr. Opin. Neurobiol.* **1999**, *9*, 336−342.
(226) Fienberg, A. A.; Hiroi, N.; Mermelstein, P. G.; Song, W. J.; Snyder, G. L.; Nishi, A.; Cheramy, A.; Ocallaghan, J. P.; Miller, D. B.; Cole, D. G. DARPP-32 − regulator of the efficacy of dopaminergic neurotransmission. *Science* **1998**, *281*, 838−842.
(227) Blitzer, R. D.; Connor, J. H.; Brown, G. P.; Wong, T.; Shenolikar, S.; Iyengar, R.; Landau, E. M. Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. *Science* **1998**, *280*, 1940−1943.
(228) Yan, Z.; Hsieh-Wilson, L.; Feng, J. A.; Mizawa, K.; len, P. B.; Enberg, A. A.; Rn, A. C.; Greengard, P. Protein phosphatase 1 modulation of neostriatal AMPA channels: regulation by DARPP-32 and spinophilin. *Nat. Neurosci.* **1999**, *2*, 13−17.
(229) Strack, S.; Wadzinski, B. E.; Ebner, F. F. Localization of the calcium/calmodulin-dependent protein phosphatase, calcineurin, in the hindbrain and spinal cord of the rat. *J. Comput. Neurol.* **1996**, *375*, 66−76.
(230) Halpain, S.; Hipolito, A.; Saffer, L. Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *J. Neurosci.* **1998**, *18*, 9835−9844.
(231) Liu, J. P.; Sim, A. T. R.; Robinson, P. J. Calcineurin inhibition of dynamin-GTPase activity is coupled to nerve terminal depolarisation. *Science* **1994**, *265*, 970−973.
(232) Yakel, J. L. Calcineurin regulation of synaptic function: from ion channels to transmitter release and gene transcription. *Trends Pharm. Sci.* **1997**, *18*, 124−134.
(233) Winder, D. G.; Mansuy, I. M.; Osman, M.; Moallem, T. M.; Kandel, E. R. Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. *Cell* **1998**, *92*, 25−37.
(234) Sim, A. T. R.; Ratcliffe, E.; Mumby, M. C.; Villa-Moruzzi, E.; Rostas, J. A. P. Differential activity of protein phosphatase types 1 and 2A in cytosolic and particulate fractions from rat forebrain. *J. Neurochem.* **1994**, *62*, 1552−1559.
(235) Sontag, E.; Nunbhakdi-Craig, V.; Bloom, G. S.; Mumby, M. C. A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **1995**, *128*, 1131−1144.
(236) Gong, C.-X.; Shaikh, S.; Wang, J.-Z.; Zaidi, T.; Grundke-Iqbal, I.; Iqbal, K. Phosphatase Activity Toward Abnormally Phosphorylated τ: Decrease in Alzheimer Disease Brain. *J. Neurochem.* **1995**, *65*, 732−738.
(237) Begum, N.; Ragolia, L. cAMP counter-regulates insulin-mediated protein phosphatase-2A inactivation in rat skeletal muscle cells. *J. Biol. Chem.* **1996**, *271*, 31166−31171.
(238) Begum, N.; Ragolia, L. Altered regulation of insulin signaling components in adipocytes of insulin-resistant type II diabetic Goto-kakizaki rats. *Metab. Clin. Exp.* **1998**, *47*, 54−62.
(239) Hansen, L.; Reneland, R.; Berglund, L.; Rasmussen, S. K.; Hansen, T.; Lithell, H.; Pedersen, O. Polymorphism in the glycogen-associated regulatory subunit of type 1 protein phosphatase (PPP1R3) gene and insulin sensitivity. *Diabetes* **2000**, *49*, 298−301.
(240) Becq, F.; Jensen, T. J.; Chang, X. B.; Savoia, A.; Rommens, J. M.; Tsui, L. C.; Buchwald, M.; Riordan, J. R.; Hanrahan, J. W. Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9160−9164.
(241) Zhu, T.; Dahan, D.; Evagelidis, A.; Zheng, S. X.; Luo, J.; Hanrahan, J. W. Association of cystic fibrosis transmembrane conductance regulator and protein phosphatase 2C. *J. Biol. Chem.* **1999**, *274*, 29102−29107.
(242) Travis, S. M.; Berger, H. A.; Welsh, M. J. Protein phosphatase 2C dephosphorylates and inactivates cystic fibrosis transmembrance conductance regulator. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11055−11060.
(243) Ludowyke, R. I.; Holst, J.; Mudge, L. M.; Sim, A. T. R. Transient translocation and activation of protein phosphatase 2A during mast cell secretion. *J. Biol. Chem.* **2000**, *275*, 6144−6152.
(244) Schriber, S. L.; Albers, M. W.; Brown, E. J. The cell cycle, signal transduction, and immunophilin ligand complexes. *Acc. Chem. Res.* **1993**, *26*, 412−420.
(245) Kaufmann, H. P.; Tobschirbel, A. An oligiopeptide from linseed. *Chem. Ber.* **1959**, *92*, 2805−2809.
(246) Husi, H.; Luyten, M. A.; Zurini, M. G. Mapping of the immunophilin-immunosuppressant site of interaction on calcineurin. *J. Biol. Chem.* **1994**, *269*, 14199−14209.
(247) Dawson, T. M.; Steiner, J. P.; Dawson, V. L.; Dinerman, J. L.; Uhl, G. R.; Snyder, S. H. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9808−9812.
(248) Dawson, T. M.; Dawson, V. L.; Snyder, S. H. A novel neuronal messenger molecule in brain: the free radical, nitric oxide. *Ann. Neurol.* **1992**, *32*, 297−311.
(249) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmol. Rev.* **1991**, *43*, 109−142.
(250) Garthwaite, J. Glutamate, nitric oxide and cell−cell signaling in the nervous system. *Trends Neurosci.* **1991**, *14*, 60−67.
(251) Dawson, V. L.; Dawson, T. M.; London, E. D.; Bredt, D. S.; Snyder, S. H. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6368−6371.

(252) Tai, P. K. K.; Chang, H.; Albers, M. W.; Schreiber, S. L.; Toft, D. O.; Faber, L. E. P59 (FK506 binding protein 59) interaction with heat shock proteins is highly conserved and may involve proteins other than steroid receptors. *Biochemistry* **1993**, *32*, 8842−8847.
(253) Begley, D. J.; Squires, L. K.; Zlokovic, B. V.; Mitrovic, D. M.; Hughes, C. C. W.; Revest, P. A.; Greenwood, J. Permeability of the blood-brain barrier to the immunosuppressive cyclic peptide cyclosporin A. *J. Neurochem.* **1990**, *55*, 1222−1230.
(254) Lopez, O. L.; Martinez, A. J.; Torre-Cisneros, J. Neuropathologic findings in liver transplantation: a comparative study of cyclosporine and FK 506. *Transplant Proc.* **1991**, *23*, 3181−3182.
(255) Travis, S. M.; Welsh, M. J. PP2C − gamma − a human protein phosphatase with a unique acidic domain. *FEBS Lett.* **1997**, *412*, 415−419.
(256) Draetta, G.; Eckstein, J. Cdc25 protein phosphatases in cell proliferation. *Biochem. Biophys. Rev. Can.* **1997**, *1332*, M53−M63.
(257) Wera, S.; Hemmings, B. A. Serine/threonine protein phosphatases. *Biochem. J.* **1995**, *311*, 17−29.
(258) O'Connor, P. M. Mammalian G_1 and G_2 phase checkpoints. *Cancer Sur.* **1997**, *29*, 151−182.
(259) Pines, J. Cyclins and cyclin-dependent kinases: Theme and variations. *Adv. Cancer Res.* **1995**, *55*, 181−212.
(260) Millward, T. A.; Zolnierowicz, S.; Hemmings, B. A. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem. Sci.* **1999**, *24*, 186−191.
(261) Stein, G. S.; Baserga, R.; Giordano, A.; Denhardt, D. T. *The molecular basis of cell cycle and growth control*; Wiley-Liss: New York, 1999.
(262) Mueller, P. R.; Coleman, T. R.; Dunphy, W. G. Cell cycle regulation of a Xenopus Wee 1-like kinase. *Mol. Biol. Cell* **1995**, *6*, 119−134.
(263) Gabrielli, B. G.; Clark, J. M.; McCromack, A. K.; Ellem, K. A. O. Hyperphosphorylation of the N-terminal domain of cdc25 regulates activity toward cyclin B1/cdc2 but not cyclin A/cdk2. *J. Biol. Chem.* **1997**, *272*, 28607−28614.
(264) Lee, T. H.; Soloman, M. J.; Mumby, M. C.; Kirschner, M. W. INH, a negative regulator of MPF, is a form of protein phosphatase 2A. *Cell* **1991**, *64*, 415−423.
(265) Roberge, M.; Tudan, C.; Hung, S. M.; Harder, K. W.; Jirik, F. R. Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. *Cancer Res.* **1994**, *54*, 6115−6121.
(266) Yamashita, K.; Yasuda, H.; Pines, J.; Yasumoto, K.; Nishitani, H.; Ohtsubo, M.; Hunter, T.; Sugimura, T.; Nishimoto, T. Okadaic acid, a potent inhibitor of type 1 and 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J.* **1990**, *9*, 4331−4338.
(267) Lazzereschi, D.; Coppa, A.; Mincione, G.; Lavitrano, M.; Fragomele, F.; Colletta, G. The phosphatase inhibitor okadaic acid stimulates the TSH-induces G1-S phase transition in thyroid cells. *Exp. Cell Res.* **1997**, *234*, 425−433.
(268) Ghosh, S.; Schroeter, D.; Paweletz, N. Okadaic acid overrides the S-phase checkpoint and accelerates progression of G2-phase to induce premature mitosis in HeLa cells. *Exp. Cell Res.* **1996**, *227*, 165−169.
(269) Durfee, T.; Becherer, K.; Chen, P. L.; Yeh, S. H.; Yang, Y.; Kilburn, A. E.; Lee, W. H.; Elledge, S. J. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **1993**, *7*, 555−569.
(270) Nelson, D. A.; Krucher, N. A.; Ludlow, J. W. High molecular weight protein phosphatase type 1 dephosphorylates the retinoblastoma protein. *J. Biol. Chem.* **1997**, *272*, 4528−4535.
(271) Yan, Y.; Mumby, M. C. Distinct roles for PP1 and PP2A in phosphorylation of the retinoblastoma protein. *J. Biol. Chem.* **1999**, *274*, 31917−31924.
(272) Afshari, C. A.; Barrett, J. C. Disruption of G_0−G_1 arrest in quiescent cells treated with phosphatase inhibitors. *Cancer Res.* **1994**, *54*, 2317−2321.
(273) Kwon, Y. G.; Lee, S. Y.; Choi, Y.; Greengard, P.; Nairn, A. C. Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2168−2173.
(274) Thompson, L. J.; Bollen, M.; Fields, A. P. Identification of protein phosphatase 1 as a mitotic lamin phosphatase. *J. Biol. Chem.* **1997**, *272*, 29693−29697.
(275) Schwartz, D. A.; Schultz, R. M. Stimulatory effect of okadaic acid, an inhibitor of protein phosphatases, on nuclear envelope breakdown and protein phosphorylation in mouse oocytes and one cell embryos. *Dev. Biol.* **1991**, *145*, 119−127.
(276) Liao, H.; Li, Y.; Brautigan, D. L.; Gundersen, G. G. Protein phosphatase 1 is targeted to microtubules by the microtubule-associated protein tau. *J. Biol. Chem.* **1998**, *273*, 21901−21908.
(277) Gurland, G.; Gundersen, G. G. Protein phosphatase inhibitors induce the selective breakdown of stable microtubules in fibroblasts and epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8827−8831.
(278) Sassoon, I.; Severin, F. F.; Andrews, P. D.; Taba, M.; Kaplan, K. B.; Ashford, A. J.; Stark, M. J. R.; Sorger, P. K.; Hyman, A. A. Regulation of *Saccharomyces cerevisiae* kinetochores by the type 1 phosphatase Glc7p. *Genes Dev.* **1999**, *13*, 545−555.
(279) Giese, G.; Weigers, W.; Kubbies, M.; Scherbarth, A.; Traub, P. Okadaic acid co-induces vimentin expression and cell cycle arrest in MPC-11 mouse plasmacytoma cells. *J. Cell. Physiol.* **1995**, *163*, 145−154.
(280) Inada, H.; Togashi, H.; Nakamura, Y.; Kaibuchi, K.; Nagata, K.; Inagaki, M. Balance between activities of rho kinase and type 1 protein phosphatase modulates turnover of phosphorylation and dynamics of desmin/vimentin filaments. *J. Biol. Chem.* **1999**, *274*, 34932−34939.
(281) Yatsunami, J.; Fujiki, H.; Suganuma, M.; Yoshizawa, S.; Eriksson, J. E.; Olsen, M. O.; Goldman, R. D. Vimentin is hyperphosphorylated in primary human fibroblasts treated with okadaic acid. *Biochem. Biophys. Res. Commun.* **1991**, *177*, 1165−1170.
(282) Ghosh, S.; Paweletz, N.; Schroeter, D. Cdc2-independent induction of premature mitosis by okadaic acid in HeLa cells. *Exp. Cell Res.* **1998**, *242*, 1−9.
(283) Ide, T.; Tsuji, Y.; Ishibashi, S.; Mitsui, Y. Reinitiation of host DNA synthesis in senescent human diploid cells by infection with simian virus 40. *Exp. Cell Res.* **1983**, *143*, 343−349.
(284) Sontag, E.; Federov, S.; Kamibayashi, C.; Robbins, D.; Cobb, M.; Mumby, M. The interaction of SV40 small tumour antigen with protein phosphatase 2A stimulates the MAP kinase pathway and induces cell proliferation. *Cell* **1993**, *75*, 887−897.
(285) Sontag, E.; Sontag, J.-M.; Garcia, A. Protein phosphatase 2A is a critical regulator of protein kinase C ζ signaling targeted by SV40 small t to promote cell growth and NF-κB activation. *EMBO J.* **1997**, *16*, 5662−5671.
(286) Carlson, S. G.; Eng, E.; Kim, E. C.; Perlman, E. J.; Copeland, T. D.; Ballermann, B. J. Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms-tumor. *J. Am. Soc. Nephrol.* **1998**, *9*, 1873−1880.
(287) Suganuma, M.; Fujiki, H.; Furuya-Suguri, H.; Yoshizawa, S.; Yasumoto, S.; Kato, Y.; Fusetani, N.; Sigimura, T. Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. *Cancer Res.* **1990**, *50*, 3521−3525.
(288) Fujiki, H.; Suganuma, M. Unique features of the okadaic acid acitivity class of tumor promotors. *J. Cancer Res. Clin. Oncol.* **1999**, *125*, 150−155.
(289) Tromovitch, T. A. Cantharidin. *J. Am. Med. Assoc.* **1971**, *215*, 640-.
(290) Sakoff, J. A.; Ackland, S. P.; Baldwin, M. L.; Keane, M. A.; McCluskey, A. Anticancer activity and protein phosphatase 1 and 2A inhibition of a new generation of cantharidin analogues. *Invest. New Drugs* In press.
(291) Jaramillo-Babb, V. L.; Sugarman, J. L.; Scavetta, R.; Wang, S.-J.; Berndt, N.; Born, T. L.; Glass, C. K.; Schönthal, A. H. Positive regulation of *cdc2* gene activity by protein phosphatase type 2A. *J. Biol. Chem.* **1996**, *271*, 5988−5992.
(292) Sakurada, K.; Zheng, B.; Kuo, J. F. Comparitive effects of protein phosphatase inhibitors (okadaic acid and calyculin A) on human leukaemia HL60, HL60/ADR and K562. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 488−492.
(293) Laidley, C. W.; Cohen, E.; Casida, J. E. Protein phosphatase in neuroblastoma cells: [^{3}H] Cantharidin binding site in relation to cytotoxicity. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 1152−1158.
(294) Takeda, Y.; Nishio, K.; Kubota, N.; Miura, K.; Morikage, T.; Ohmori, T.; Kudoh, S.; Niitani, H.; Saijo, N. Establishment of a human small-cell lung cancer subline resistant to okadaic acid. *Int. J. Cancer* **1994**, *58*, 882−890.
(295) Tohda, H.; Takao, M.; Kikuchi, A.; Yasumoto, T.; Yasui, A. Unstable expression of the multi-drug resistant phenotype in chinese hamster ovary cells resistant to okadaic acid. *Biochem. Biophys. Res. Commun.* **1997**, *232*, 398−402.
(296) Leopold, W. R.; Shillis, J. L.; Mertus, A. E.; Nelson, J. M.; Roberts, B. J.; Jackson, R. C. Anticancer activity of the structurally novel antibiotic CI-920 and its analogues. *Cancer Res.* **1984**, *44*, 1928−1932.
(297) Scheithauer, W.; Von Hoff, D. D.; Clark, G. M.; Shillis, J. L.; Elslager, E. F. In vitro activity of the novel antitumour antibiotic fostriecin (CI-920) in a human tumour cloning assay. *Eur. J. Cancer Clin. Oncol.* **1986**, *22*, 921−926.
(298) Fry, D. W.; Besserer, J. A.; Boritzki, T. J. Transport of the antitumour antibiotic CI-920 into L1210 leukaemis cells by the reduced folate carrier system. *Cancer Res.* **1984**, *44*, 3366−3370.
(299) de Jong, R. S.; de Vries, E. G. E.; Mulder, N. H. Fostriecin: a review of the preclinical data. *Anti-Cancer Drugs* **1997**, *8*, 413−418.
(300) de Jong, R. S.; de Vries, E. G. E.; Meijer, S.; de Jong, P. E.; Mulder, N. H. Renal toxicity of the anticancer drug fostriecin. *Cancer Chemother. Pharmacol.* **1998**, *42*, 160−164.

(301) de Jong, R. S.; Mulder, N. H.; Uges, D. R. A.; Sleijfer, D. T.; Hoppener, F. J. P.; Groen, H. J. M.; Willemse, P. H. B.; van der Graaf, W. T. A.; de Vries, E. G. E. Phase 1 and pharmacokinetic study of the topoisomerase II catalytic inhibitor. *Br. J. Cancer* **1999**, *79*, 882−887.
(302) Einbinder, J. M.; Parshley, M. S.; Walzer, R. A.; Sanders, S. L. The effects of cantharidin on epithelial cells in tissue culture. *J. Invest. Dermatol.* **1969**, *53*, 291−303.
(303) Wang, G.-S. Medical uses of mylabris in ancient china and recent studies. *J. Ethnopharmacol.* **1989**, *26*, 147−162.
(304) Wu, J. Z.; Situ, Z. Q.; Chen, J. Y.; Liu, B.; Wang, W. Chemosensitivity of salivary gland and oral cancer cell lines. *Chin. Med. J.* **1992**, *105*, 1026−1028.
(305) Jiang, T.-L.; Salmon, S. E.; Liu, R. M. Activity of camptothecin, harrington, cantharidin and curcumae in the human stem cell assay. *Eur. J. Clin. Oncol.* **1983**, *19*, 263−270.
(306) Sieder, S.; Richter, E.; Becker, K.; Heins, R.; Steinfelder, H. J. Doxorubicin-resistant LoVo adenocarcinoma cells display resistance to apoptosis induction by some but not all inhibitors of ser thr phosphatases 1 and 2A. *Toxicology* **1999**, *134*, 109−115.
(307) Yi, S.; Wass, J.; Vincent, P.; Iland, H. Inhibitory effect of norcantharidin on K562 human myeloid leukaemia cells in vitro. *Leuk. Res* **1991**, *15*, 883−886.
(308) Liu, X.-H.; Balzsek, I.; Comisso, M.; Legras, S.; Marion, S.; Quittet, P.; Anjo, A.; Wang, G.-S. Effects of norcantharidin, a protein phosphatase type-2A inhibitor, on the growth of normal and malignant haemopoietic cells. *Eur. J. Cancer* **1995**, *31A*, 953−963.
(309) Cheng, A.; Balczon, R.; Zuo, Z.; Koons, J. S.; Walsh, A. H.; Honkanen, R. E. Fostriecin mediated G_2-M-phase growth arrest correlates with abnormal centrosome replication, the formation of aberrant mitotic spindles, and the inhibition of serine/threonine protein phosphatase activity. *Cancer Res.* **1998**, *58*, 3611−3619.
(310) Sakoff, J. A.; McCluskey, A.; Sim, A. T. R.; Stewart, J. F.; Ackland, S. P. Selective solid tumor anticancer activity of cantharidin (Spanish Fly) analogues. *Proc. Am. Assoc. Cancer Res.* **2000**, *41*, 770.
(311) Nakamura, K.; Antoku, S. Enhancement of X-ray cell killing in cultured mammalian cells by the protein phosphatase inhibitor calyculin A. *Cancer Res.* **1994**, *54*, 2088−2099.
(312) Fujita, M.; Seta, C.; Fukoda, J.; Kobayashi, S.; Haneji, T. Induction of apoptosis in human oral squamous carcinoma cell lines by protein phosphatase inhibitors. *Oral Oncol.* **1999**, *35*, 401−408.
(313) Kikuchi, K.; Shima, H.; Mitsuhashi, S.; Suzuki, M.; Oikawa, H. New apoptosis-inducing agents: thyrsiferyl 23-acetate, tautomycin and related compounds. *Drugs Future* **2000**, *25*, 501−507.
(314) Hong, C. Y.; Hunag, S. C.; Lin, S. K.; Lee, J. J.; Chueh, L. L.; Lee, C. H. K.; Lin, J. H.; Hsiao, M. Norcantharidin-induced post-G(2)/M apoptosis is dependent on wild-type p53 gene. *Biochem. Biophys. Res. Commun.* **2000**, *276*, 278−285.
(315) Fladmark, K. E.; Brustugun, O. T.; Hovland, R.; Boe, R.; Gjertsen, B. T.; Zhivotovsky, B.; Doskeland, S. O. Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. *Cell Death Differ.* **1999**, *6*, 1099−1108.
(316) Morisaki, T.; Matsunaga, H.; Beppu, K.; Ihara, E.; Hirano, K.; Kanaide, H.; Mori, M.; Katano, M. A combination of cyclosporin-A (CsA) and interferon gamma (INF-gamma) induces apoptosis in human gastric carcinoma cells. *Anticancer Res.* **2000**, *20*, 3363−3373.
(317) Kleinberger, T. Induction of apoptosis by adenovirus E4orf4 protein. *Apoptosis* **2000**, *5*, 211−215.
(318) Marcellus, R. C.; Chan, H.; Paquette, D.; Thirlwell, S.; Boivin, D.; Branton, P. E. Induction of p53-independent apoptosis by the adenovirus E4orf4 protein requires binding to the B alpha subunit of protein phosphatase 2A. *J. Virol.* **2000**, *74*, 7869−7877.
(319) Ayllon, V.; Martinez, C.; Barcia, A.; Cayla, X.; Rebollo, A. Protein phosphatase 1 alpha is a Ras-activated Bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. *EMBO J.* **2000**, *19*, 2237−2246.
(320) Morana, S. J.; Wolf, C. M.; Li, J. F.; Reynolds, J. E.; Brown, M. K.; Eastman, A. The involvement of protein phosphatases in the activation of ICE/CED-3 protease, intracellular acidification, DNA digestion, and apoptosis. *J. Biol. Chem.* **1996**, *271*, 18263−18271.
(321) Asai, A.; Qiu, J.; Narita, Y.; Chi, S.; Saito, N.; Shinoura, N.; Hamada, H.; Kuchino, Y.; Kirino, T. High level calcineurin acitivity predisposes neuronal cells to apoptosis. *J. Biol. Chem.* **1999**, *274*, 34450−34458.
(322) Puntoni, F.; Villa-Moruzzi, E. Protein phosphatase-1 activation and association with the retinoblastoma protein in colcemid-induced apoptosis. *Biochem. Biophys. Res. Commun.* **1999**, *266*, 279−283.
(323) Salomoni, P.; Condorelli, F.; Sweeney, S. M.; Calabretta, B. Versatility of BCR/ABL-expressing leukemic cells in circumventing proapoptotic BAD effects. *Blood* **2000**, *96*, 676−684.
(324) Wang, H. G.; Pathan, N.; Ethell, I. M.; Krajewski, S.; Yamaguchi, Y.; Shibasaki, F.; McKeon, F.; Bobo, T.; Franke, T. F.; Reed, J. C. Ca^{2+}-induced apoptotic cell death through calcineurin dephosphorylation of BAD. *Science* **1999**, *284*, 339−343.
(325) Ruvolo, P. P.; Deng, X.; Ito, T.; Carr, B. K.; May, W. S. Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. *J. Biol. Chem.* **1999**, *274*, 20296−20300.
(326) Haldar, S.; Jena, N.; Croce, C. M. Inactivation of bcl-2 by phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4507−4511.
(327) Nuydens, R.; Dispersyn, G.; Van den Kieboom, G.; de Jong, M.; Connors, R.; Ramaekers, F.; Borgers, M.; Geerts, H. Bcl-2 protects against apoptosis-related microtubule alterations in neuronal cells. *Apoptosis* **2000**, *5*, 43−51.
(328) Petit, P. X.; Goubern, M.; Diolez, P.; Susin, S. A.; Zamzami, N.; Kroemer, G. Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. *FEBS Lett.* **1998**, *426*, 111−116.
(329) Sakoff, J. A.; Ackland, S. P. Thymidylate synthase inhibition induces S-phase arrest, biphasic mitochondrial alterations and caspase dependent apoptosis in leukaemia cells. *Cancer Chemother. Pharmacol.* **2000**, *46*, 477−487.

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