Small Molecule Inhibitors of Serine/Threonine Protein Phosphatases

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Abstract: Serine/threonine protein phosphatases have long been ignored as potential therapeutic targets for two reasons, one the biochemical significance of these proteins has not been appreciated and two, many natural protein phosphatase inhibitors are potent toxins and are considered unsuitable for clinical use. This review outlines the biochemical role of this protein family in cancer, cystic fibrosis, immunosuppression and, cardiac and neurological disorders. Particular emphasis is also given to the synthesis of selective small molecule inhibitors and their clinical exploitation.

INTRODUCTION

Regulation of cellular processes by modification of the levels of phosphorylated proteins is fundamental to a large number of, if not all, cellular functions. The actual phosphorylation level is the result of a delicate balance between phosphatases and kinases. Kinases transfer a phosphate from ATP to a protein, typically phosphorylation at a serine, threonine or tyrosine residue. Phosphatases, on the other hand remove the phosphate group, i.e. dephosphorylate the protein. In contrast to the multitude of protein kinases that have been discovered, relatively few protein phosphatases are known. In this regard protein phosphatases were often considered as the poorer cousins of kinases. However, it is now 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. In many ways, the phosphorylation-dephosphorylation cycle can be regarded as a molecular "on-off" switch, although this is probably an over simplification of the processes involved. The end result of these associated pathways is the regulation of many different cellular processes, as diverse as glycogen metabolism, calcium transport, muscle contraction, gene expression, protein synthesis, intracellular transport, phototransduction, cell cycle progression and apoptosis [1-3].

CLASSIFICATION

Three distinct classes of protein phosphatases exist: tyrosine-specific, serine/threonine-specific, and dual-specificity phosphatases. Since these classes have very distinct functions this review will focus on the structure, function and inhibition of the serine/threonine class. Traditionally, the protein phosphatases (PPs) that catalyse the dephosphorylation of serine and threonine residues have been classified into four subtypes based on their biological characteristics, sensitivities to specific inhibitors, and substrate specificity including PP1, PP2A, PP2B (calcineurin, Ca^{2+}/calmodulin dependent protein phosphatase), and PP2C (ATP / Mg^{2+} dependent protein phosphatase), multiple isoforms of each also exist [1,3-7]. The primary amino acid sequences of PP1, PP2A, and PP2B are similar, with PP1 and PP2A sharing 43% sequence identity overall and 50% identity for residues 23-292; whereas PP2C is structurally distinct and belongs to a completely different gene family. Other phosphatases have been identified including PP4, PP5, PP6 and PP7. PP4 (formally known as PPX) and PP6 are structurally related to PP2A, sharing 65 and 57% amino acid homology. PP4 is 45% identical to PP1, but only in its catalytic domains. PP5 contains a catalytic domain common to PP1, PP2A, PP2B, PP4 and PP6 family of enzymes and an extended N-terminal domain containing four 34-amino acid repeat motifs. PP7 contains a catalytic core domain similar to the other phosphatases, but has unique N- and C-terminal regions and shares <35% identity with other known protein phosphatases [8]. Other recent additions to these classes include relatives of PP1: PPZ1, PPZ2, PPQ; relatives of PP2A: PPV, PPG; and a new PP2B, rdgC. Despite this wide range of protein phosphatases, the majority of phosphatase activity within the cell is attributable to PP1 and PP2A [9,10].

STRUCTURE AND FUNCTION

(a) PP1 and PP2A

Both PP1 and PP2A possess a complex holoenzyme structure, the catalytic subunits (PP1c, 37kDa and PP2Ac 36kDa) of which are structurally related sharing 50% amino

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acid identity. Interestingly, PP1 shows ca. 90% sequence identity across mammalian and drosophila species, representing one of the most conserved classes of enzymes known. The catalytic subunits also associate with additional regulatory units that attenuate the substrate specificity of PP1 and PP2A, giving rise to specific sub-cellular localisations. PP2A typically exists as a heterotrimer comprising three subunits: A, B and C; additionally there are a number of different A and C units known. A couple of B units have also been identified and these units are believed responsible for the distinct substrate specificity observed. PP1 and PP2A are also stringently regulated by a number of endogenous protein inhibitors. PP1 is specifically inhibited by Inhibitor-1 (I-1), Inhibitor-2 (I-2), dopamine and cAMP-regulated phosphoprotein (DARPP-32), and nuclear inhibitor of protein phosphatase 1 (NIPP-1). PP2A is specifically inhibited by putative histocompatability leukocyte antigens class II associated protein I (PHAP-I, or I-1PP2A) and II (PHAP-II, or I-2PP2A, SET). Further regulation occurs indirectly via activation and deactivation of secondary messenger systems [11,12].

Gene mutations in PP1 or PP2A lead to a variety of defects in mitosis, indicating an important role for these phosphatases in the cell cycle [1]. In particular, PP1 yeast mutants are unable to complete anaphase successfully or instigate chromosome segregation, while in drosophila such mutants die at the larval-pupal boundary with defective spindle organisation, abnormal sister chromatid segregation, hyperploidy, and excessive chromosome condensation. Not surprisingly, PP1 overexpression in yeast is lethal. PP2A yeast mutants are not viable, however, mutants lacking only one of the PP2A subunits display defects in cell septation and separation, and become multinucleated, while in drosophila abnormal anaphase resolution occurs. Both phosphatases have been shown to control key signal transduction mechanisms involved in cell cycle progression, with PP1 and PP2A often classified as negative regulators of the cell cycle [1,2,13-16]. This negative regulation involves the inactivation of the retinoblastoma protein (pRb), and cyclin dependent kinases (cdk). The latter can be achieved by directly dephosphoryling cdks or indirectly by influencing upstream and downstream kinases/phosphatases. Other mitotic events are also controlled by PP1 and PP2A including chromosome condensation, nuclear membrane disintegration, reorganisation of cytoplasmic microtubules, spindle formation, chromatid separation, nuclear membrane reassembly and cytokinesis [17-24].

The role of PP1 and PP2A as negative regulators of the cell cycle is exemplified by the ability of various inhibitors including okadaic acid, calcyculin A, microcystin, cantharidin and fostriecin to accelerate and enhance cell cycle progression [25,26]. Not surprisingly, many PP1 and PP2A inhibitors (okadaic acid, calyculin A, microcystin) are classified as tumour promoters [27]. However, PP1 and PP2A inhibitors are also potent anticancer drugs and mediate their effects by prematurely activating cell cycle progression and by inhibiting vital mitotic processes [28-30]. Of particular interest are cantharidin and its analogues, [31,32] and fostriecin, both of which have been used clinically for the treatment of cancers [33-35].

(b) PP2B

PP2B (calcineurin, Ca^{2+} / calmodulin dependent protein phosphatase) is a heterodimer comprising A and B subunits that share some overlapping substrate specificity with both PP1 and PP2A [1]. Calcineurin A, a 60kDa catalytic subunit, has ca 40% homology with the catalytic subunits of PP1 and PPA, but contains an additional 170 C-terminal amino acids, the calmodulin binding domain. The B subunit has a molecular weight of 19kDa and possesses four Ca^{2+}-binding sites. Calcineurin is modulated by Ca^{2+}, calmodulin, and possibly by FK506-binding proteins and the cyclophilins [36,37].

PP2B shows relatively low activity in the heart, spleen, liver and testes but is highly enriched in brain tissue, particularly in the striatum and hippocampus and localised along dendritic spines. This is consistent with its proposed role in memory by modulating long term potentiation and long term depression of synaptic efficacy [38,39]. Over expression of PP2B in transgenic mice produced deficits in long-term memory and a constraint on long term potentiation. Other neurological studies have shown PP2B to be abundantly expressed in areas of the brain, which are vulnerable to stroke, epilepsy and neurodegenerative diseases. It has been suggested that high level activity of PP2B predisposes neuronal cells to apoptotic cell death and that PP2B inhibitors reduce such susceptibility [40]. Some of the neurological and physiological and abnormalities of Downs syndrome may also be attributed to PP2B inhibition. In this context, DSCR1 is a family of proteins coded by chromosome 21, that are overexpressed in the brain and striated muscles of Down syndrome patients, and which inhibit PP2B signalling pathways [41-43].

PP2B also plays a key role in inflammation and immunosuppression. Inhibition of PP2B by cyclosporin A and FK506 suppresses the production of IL-2 and other cytokines in activated T cells and these two agents have become the main immunosuppressive agents used in the prevention of allograft organ rejection [44]. Cyclosporin A inhibits PP2B by sequestering it into a molecular complex with cyclophilin. FK506 inhibition requires the formation of a molecular complex with the intracellular FK506-binding-protein-12, which interferes with the ability of PP2B to interact with its target proteins [45]. PP2B also appears to function in the long-term maintenance of cardiac hypertrophy or myopathic disease states [46]. Consequently, the PP2B inhibitors, cyclosporin A and FK506, protect against heart ischemia [47].

PP2B has also been implicated in controlling membrane channels and pumps. PP2B is involved with the intracellular signalling action of insulin on single chloride channel conductance in renal epithelium [48]. The role of PP2B in regulating cellular calcium channels may explain the occurrence of renal failure associated with FK506 treatment [49]. PP2B has also been implicated in controlling the activity of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Dephosphorylation of this channel results in inactivation and excessive mucous secretion in airway epithelia [50]. Finally, activity of the multidrug-resistance transmembrane

pump, P-glycoprotein, is attenuated by FK506 and cyclosporin [51-53]. Multidrug resistance is often mediated by p-glycoprotein, which actively pumps compounds out of the cell.

(c) PP2C

PP2C is a monomeric Mg^{2+}/Mn^{2+} dependent protein that has no homology with any of the other known phosphatases [54], and is insensitive to okadaic acid and other naturally occurring protein phosphatase inhibitors. However, it does display a degree of overlapping substrate specificity particularly with PP1 and PP2A. Multiple isoforms (α, β, χ, δ) of PP2C have been identified in humans, yeast and mice [4,5,55-57]. PP2Cs are widely expressed but most abundant in heart and skeletal muscle [4,6].

Multiple genes code for PP2C [56,58], allowing for tissue specific expression [57]. The deletion of one of these genes is not lethal. However, yeast mutant lacking PP2C show growth abnormalities and are more sensitive to stress conditions including heat shock, osmostress, oxidative stress and nutrient starvation [56,59,60]. In normal eukaryotic cells the environmental stress response pathway is down regulated by the PP2C family (also known as PPM). This signal transduction pathway is a protein kinase cascade involving key mitogen-activated protein kinases (MAPK, p38) and well as upstream MAPK kinases (MKK3b, MKK4, MKK6b, MKK7), and even MAPK kinase kinases, together with downstream kinases which are all activated by protein phosphorylation and subsequently inhibited by PP2C enzymes [59,61-63]. PP2C has also been shown to negatively regulate the cell cycle in a manner similar to that of PP1 and PP2A via the dephosphorylation and deactivation of cdks [64]. Not surprisingly, overexpression of PP2Cδ in yeast induces an early S-phase cell cycle arrest resulting in cell death [55].

As with PP2B, PP2C has also been implicated in the respiratory disease cystic fibrosis and the dephosphorylation of CFTR, however, PP2C appears to be the primary phosphatase. This disease is characterised by abnormalities in CFTR and evidence shows that PP2C is the phosphatase that dephosphorylates and inactivates CFTR in airway and intestinal epithelia [65-67]. It has been suggested that PP2C inhibitors could be of therapeutic value in the treatment of this disease [65].

INHIBITION OF PROTEIN PHOSPHATASES

An interesting link between PP1 and PP2A is their shared sensitivity, presumably as a consequence of their shared homology, towards a structurally diverse family of natural products: the okadaic acid class of compounds, exemplified by those illustrated in Figure 1. These compounds are potent, competitive inhibitors of both PP1 and PP2A and include okadaic acid (**1**) [68], tautomycin (**2**) [69], the microcystins, (microcystin-LR (**3**)) [70] and the

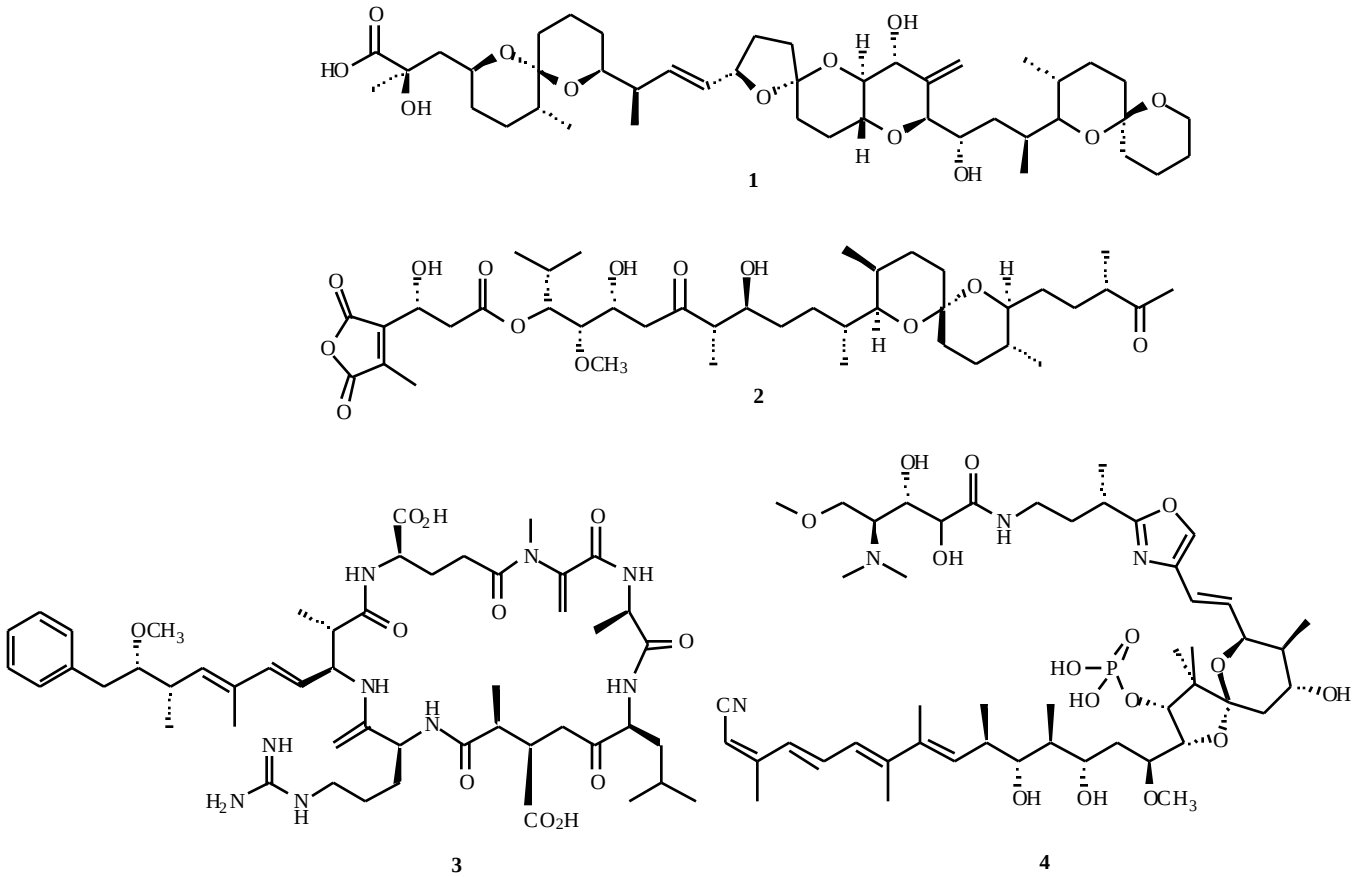


Fig. (1). The okadaic acid class of compounds.

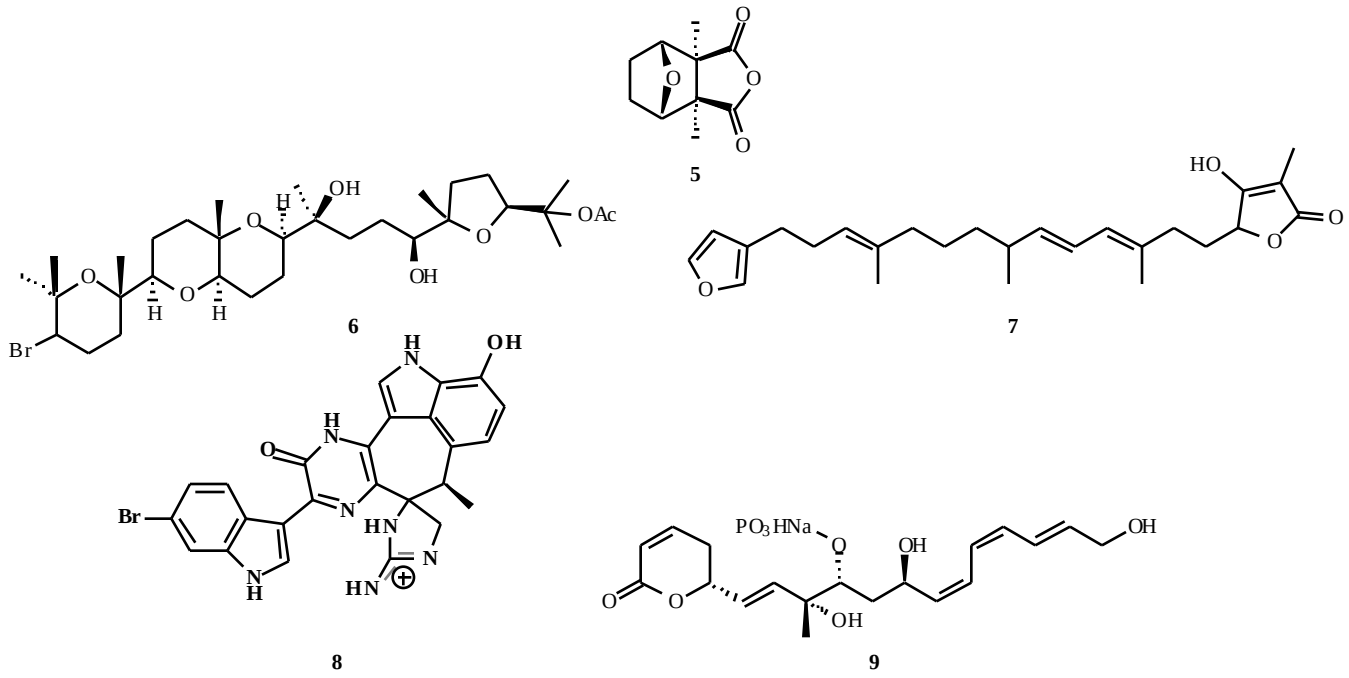


Fig. (2). Small molecule inhibitors of serine/threonine protein phosphatase.

calyculins A-H (calcyculin-A (**4**)) [28]. The okadaic acid class of compounds, and their inhibition of serine/threonine protein phosphatases have been subject of recent excellent review by Chamberlin [71], and the modelling aspects of their modes of binding have also been reviewed by Chamberlin [72], Armstrong [73] and Holmes [74]. The structural complexity of these naturally occurring toxins means that they are exciting targets for the synthetic chemist, whilst their biological properties entice the interests of medicinal chemists and their cohorts.

In the past few years there have been an increasing number of literature reports of small molecule inhibitors of protein phosphatases: cantharidin (**5**)[75], thyrsferyl 23-acetate (**6**)[76], isopalinurin (**7**)[77], dragmacidins (dragmacidin E (**8**))[78], and fostriecin (**9**)[26], see Figure 2.

The reason for such interest is that these molecules are showing greater promise for selective inhibition of protein phosphatases. This is particularly evident with fostriecin being 40,000 fold PP2A selective, with an IC_{50} versus PP2A of 3.4 nM [79], see Table 1.

Of all the known inhibitors of serine/threonine protein phosphatases, cantharidin offers the simplest structure for subsequent modification, as such there have been a number of reports of synthetic modifications and biological data. However, this data is difficult to rationalise due to the varying inhibition values reported, which for PP2A range from 40 nM to 8 μM. It is possible that a combination of differing assay methodologies, variation in enzyme preparation (recombinant, chicken, mouse) or purity or substrate concentration and/or the purity of the material being assayed accounts for the observed differences in the inhibition reported with cantharidin, see table 2 [31,109,110,113]. As a result, in this short review we have attempted to rationalise the relative potency of all the known cantharidin analogues with a view toward gaining a better

Table 1. Inhibition of Serine/Threonine Protein Phosphatases 1 and 2A by Various Natural Toxins (IC_{50} nM)

Inhibitors	Protein Phosphatase Inhibition (IC_{50} nM)			
	PP1	PP2A	Selectivity (PP1/PP2A)	Refs
Okadaic Acid	42	0.51	82	[71]†
Calyculin A	2	0.5	4	[71]†
Tautomycin	0.3	1	0.3	[71]†
Microcystin-LR	1.7	0.04	43	[71]†
Fostriecin	131000	3.4	39,000	[71]†
Cantharidin *	1300	130	10	[31,110,113,114]
Thyrsiferyl-23-acetate	$>1x10^6$	$4\text{-}16x10^6$	-	[71]†

* Average of [31,110,113,114]. † references cited therein

understanding of potential synthetic modifications that will allow the development of more potent and selective inhibitors.

Table 2. Inhibition of Serine/Threonine Protein Phosphatases by Cantharidin

Cantharidin Protein Phosphatase Inhibition[a]			
PP1	**PP2A**	**PP2B**	**Ref**
-	0.065 μM	10.8 μM	[110]
1.7 μM	0.16 μM	>800 μM	[113]
473 nM	40 nM	>30,000 nM	[114]
1.78μM	0.26μM	-	[31]
-	92-95%, 4-8 nM	-	[75]
-	95%, 8 μM	-	[111,112]
82%	81%	18%	[115]

[a] all reports indicate that PP2C inhibition is >100μM

Little other structure activity work has been reported, although selected modifications of fostriecin have been carried out. No phosphatase inhibition data was reported; however data pertaining to its potency as an anti-tumour agent was recorded [35], see table 3. In this context, 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.

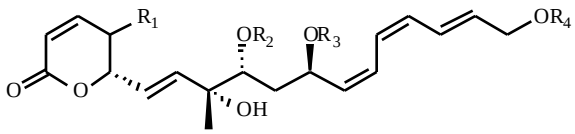
CANTHARIDIN

(a) Biological Effects

The Meloidae family of Coleoptera (beetles) has been known since antiquity to produce a potent defensive agent [80]. This agent is now known as cantharidin (exo, exo-2, 3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride) (**5**) and was first isolated from blister beetles by Robiquet in 1810 [81]. Adult beetles deter attacks of many predators by discharging droplets of cantharidin-laden blood reflexively from their hind leg joints. During mating the male also deposits a spermatophore containing many micrograms of cantharidin in the females spermatophoral receptacle. This copulatory gift is then used to protect the fertilised eggs from predation [82]. Cantharidin is found in over 1500 species of 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 [80]. In Chinese medicine cantharidin is also known as Mylabris which is the dried body of the *Mylabris* beetle.

Cantharidin is a toxin. Exposure of the skin to cantharidin induces blisters and the separation of cells known as acantholysis [83], hence the term blister beetles. Ingestion of cantharidin produces a large variety of physiological symptoms, the degree of which varies with the magnitude of exposure. Generally, an injected dose of between 10-65 mg is lethal within 12-24 h although some patients have survived as long as 7 days [84,85]. Cantharidin poisoning is characterised by dysphagia, nausea, hematemesis (vomiting of bloodstained material), severe abdominal pain, mucosal erosion and haemorrhage of the gastrointestinal tract, diarrhoea with passage of stools containing blood and mucous, lumbar pain, urinary tract

Table 3. Structural Modifications of Fostriecin and their Antitumour (IC_{50}) Effects in L1210 (leukaemia) and HCT-8 (colon) Tumour Cell Lines [35].



Entry	R_1	R_2	R_3	R_4	IC_{50}‡ (μg / mL)	
					L1210	**HCT-8**
1	H	PO_3^{2-}	H	OH	0.21	2.3
2	H	PO_3^{2-}	H	H	0.22	1.5
3	OH	PO_3^{2-}	H	OH	0.83	4.2
4	H†	PO_3^{2-}	H	OH	>1.0	56
5	H	H	H	OH	2.5	3.7

‡IC_{50} concentration of drug that will reduce tumour cell growth to 50% of the control value.

†Open lactone

urgency and frequency, hematuria, dysuria, oliguria, uterine bleeding or abortion, painful priapism, convulsions, shock, coma and death [86-90]. Renal failure due to tubular necrosis and glomerular destruction characterises cantharidin poisoning. Hepatic damage [91], neurological complications including paralysis [89] and myocardial damage [92-94] have also been observed.

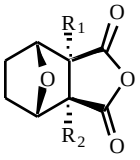
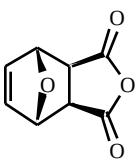
As a result of the irritation of the urogenital tract, severe priapism in men and pelvic engorgement of women occurs, which explains the perceived, although incorrect, aphrodisiac qualities of cantharidin (Spanish fly). While cantharidin is obviously toxic, poisoning and death related to the accidental exposure of cantharidin is rare. Case histories have shown poisoning of small children eating blister beetles [86,88], and fatalities due to use of the drug as an abortifacient [95]. However, the vast majority of poisonings and fatalities related to this compound have occurred as a result of its promiscuous use as an aphrodisiac [84-87,90,93,96,97]. A veterinary concern with cantharidin poisoning also exists with farm animals ingesting alfalfahay contaminated with blister beetles [98-100].

(b) Therapeutic Uses

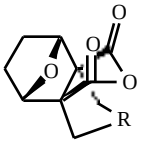
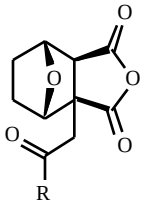
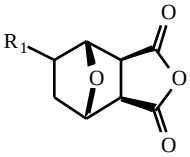
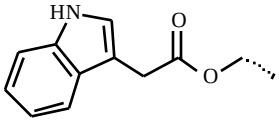
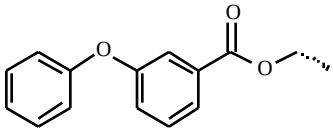
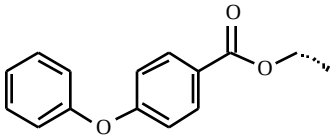
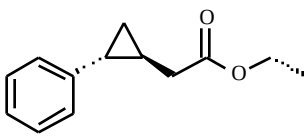
Cantharidin has been used as a medicinal agent for over 2000 years and is listed as a drug under the name of Mylabris in the medical monograph *Materia Medica* published in 77 AD [33]. Some of the most ancient Chinese prescriptions (306-168 BC) refer to the use of Mylabris for the treatment of furuncles and piles [33]. Hippocrates also describes cantharidin in the treatment of dropsy [84]. Other early research articles describe cantharidin as a blistering agent used in the treatment of pleurisy and pericarditis [101]. In more recent times cantharidin has been used topically (0.7%) in the treatment of warts. Interestingly, not a single case of systemic toxicity has been observed with its dermatological use [102]. When applied topically cantharidin is also used as a biochemical tool. The induced blister fluid is analysed to test the penetrating and accumulative capacity of various drugs such as antibiotics [103]. Cantharidin is also exploited to test the anti-inflammatory capacity of various drugs [104]. Of the medicinal uses of cantharidin the most important is its anticancer activity.

The first recorded use of cantharidin as an anti-cancer agent was in 1264 [33]. In more recent studies including our own, cantharidin has been shown to be active in cervical, tongue, ginival, neuroblastoma, bone, leukaemia, ovarian, and colon cancer cell lines cells [31,33,105,106]. Cantharidin reduces cell viability in a time-, concentration- and cell line-dependent manner with IC_{50} values generally in the low μM range; i.e. in neuroblastoma cells the IC_{50} value

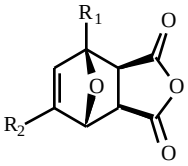
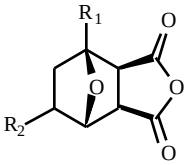
Table 4. Skeletally Modified Cantharidin Analogues and Inhibition of PP1, PP2A and PP2B

Entry	Compound	R_1	R_2	Protein Phosphatase Inhibition			Ref
				PP1	PP2A	PP2B	
1		CH_3	H	-	22-51%, 19-50 nM	-	[75]
				656 nM	120 nM	-	[114]
				[^{3}H]CA, IC_{50}=ND	Mouse ip, LD_{50}=3.1 mg/kg	[^{3}H]ETA, IC_{50}=12 nM	[114]
2		H	H	93%	90%	39%	[115]
				-	88%, 70μM	-	[112]
				-	100%	-	[111]
				-	0.2μM	3.33μM	[110]
				[^{3}H]CA, IC_{50}=1800nM	Mouse ip, LD_{50}=4.0 mg/kg	[^{3}H]ETA, IC_{50}=26nM	[114]
3				91%	90%	46%	[115]
				-	95%, 8μM	-	[111,112]
				-	97%	-	[115]

(Table 4). contd.....

Entry	Compound	R_1	R_2	Protein Phosphatase Inhibition			Ref
				PP1	PP2A	PP2B	
4		S		-	10 nM	-	[109]
5		CH_2		-	3 nM	-	[109]
6		OH		-	45 nM	-	[109]
7		$OCH_2C_6H_9$		-	42nM	-	[109]
8		$OCH_2C_6H_4$-4-OCH_3		-	48 nM	-	[109]
9		NHC_4H_9-*n*		-	251 nM	-	[109]
10		NHC_7H_{15}-*n*		-	206 nM	-	[109]
11		$PhCO_2CH_2$		-	-	11.0μM	[116]
12		$PhCH_2CO_2CH_2$		-	-	7.4μM	[116]
13		$PhCH_2CH_2CO_2CH_2$		-	-	3.7μM	[116]
14		$Ph(CH_2)_3CO_2CH_2$		-	-	1.2μM	[116]
15		$Ph(CH_2)_4CO_2CH_2$		-	-	2.3μM	[116]
16		*c*-$C_6H_{11}(CH_2)_3CO_2CH_2$		-	-	1.0μM	[116]
17				-	-	2.0μM	[116]
18				-	-	1.8μM	[116]
19				-	-	1.4μM	[116]
20				4.0 μM	-	0.5μM	[116]

(Table 4). contd.....

Entry	Compound	R_1	R_2	Protein Phosphatase Inhibition			Ref
				PP1	PP2A	PP2B	
21		CH_2OAc	H	7%	2%	14%	[115]
22		CH_2OBz		5%	6%	10%	[115]
23		H	CH_2OAc	58%	60%	27%	[115]
24		H	CH_2OBz	57%	89%	33%	[115]
25		CH_2OAc	H	23%	7%	30%	[115]
26		H	CH_2OAc	94%	81%	24%	[115]
27		H	CH_2OBz	76%	81%	44%	[115]

after 24h incubation was 4.5μM decreasing progressively to 1.3μM by 120h [106], we have reported similar values of 6-11μM after 72h in ovarian, bone and colon cancer cells [31]. Cantharidin is also active in ovarian, melanoma, and epidermoid carcinoma (originally from primary tumour of the larynx) biopsies, but less active in cancer biopsies of lung, adenocarcinoma, pancreas, breast, cervix, mesothelioma or sarcoma tumours [107]. Interestingly, clinical trials involving cantharidin have shown this agent to stimulate the production of white blood cells by the bone marrow. 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 kidney toxicity of this drug has prevented its use in mainstream oncology.

Norcantharidin (**10**), the demethylated analogue of cantharidin also possesses anticancer activity and stimulates the bone marrow, however, the nephrotoxicity associated with cantharidin treatment is absent [33,108]. Norcantharidin is active *in vitro* against several tumour cell lines including cervical, hepatoma, ovarian, laryngocarcinoma, colon, osteocarcinoma, and leukaemia cell lines [31,33,108]. However, our own studies have shown norcantharidin to be approximately 10 fold less cytotoxic than cantharidin in many of these cell lines. Norcantharidin has also been used *in vivo* in the treatment of primary hepatoma, oesophageal, gastric and cardia carcinomas [33].

(c) Structure Activity Relationships

Structural modifications of cantharidin can be broken down into several categories; skeletal modifications, that is addition of alkyl side-chains to the bicyclo[2.2.1]heptane framework (Table 4); modification of the anhydride bridge including atom substitution and ring opening (Table 5); modification of the 7-oxo-bridge, and multiple modifications.

SKELETAL MODIFICATIONS

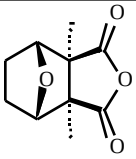
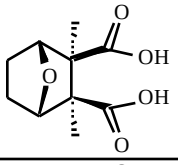
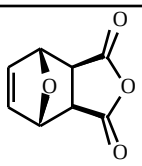
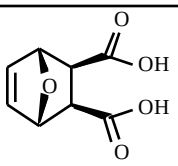
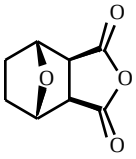
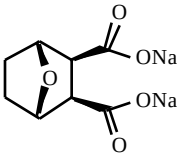
A significant proportion of the synthetic work associated with cantharidin has involved removal of the 2,3-dimethyl substituents (either one: palsonin; or both norcantharidin). As can be seen in table 4, neither of these modifications have a significant effect on the inhibitory properties of these analogues.

However, the modifications do simplify the chemistry required for their synthesis. Other skeletal modifications, such as additional substituents at the 5- and 6- positions adversely affect interaction at both PP1 and PP2A whilst enhancing inhibition at PP2B (Table 4, entries 23 – 27). Indeed modifications at the 5-endo position result in a 20-fold increase in inhibition of PP2B and an increase in PP2B vs. PP2A selectivity, such that entry 20 (table 4) now shows 8-fold PP2B selectivity. In the case of PP2A activity a recent report by Laidley et al [109] suggests that there is a significant binding pocket at C-2 able to accommodate large groups with no loss of inhibition (see table 4 entries 6 - 10).

ANHYDRIDE MODIFICATIONS

As can be seen from table 5a the PP2A inhibition values differ when the anhydrides (entries 1 and 2) are compared with those of their ring opened dicarboxylic acid partners. The general trend is that the dicarboxylic acid partner

Table 5a. Effects of Anhydride to Dicarboxylic Acid Modification on the Inhibition of PP2A

Entry	Anhydride	Inhibition of PP2A[b]	Carboxylic acid	Inhibition of PP2A	Ref.
1		IC_{50} = 40 nM 92-95%		IC_{50} = 53 nM 92-95%	[75] [113]
2		97%		80%	[112]
3		IC_{50} = 0.2μM		IC_{50} = 0.17 μM	[110]

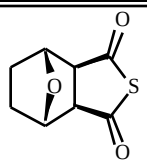
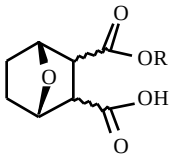
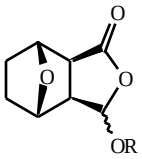
[b]Inhibitory values expressed as a percentage were measured at 100 μM compound concentration. Other values are IC_{50} values.

exhibits poorer PP2A inhibition values. This is also observed in multiply modified analogues [75,113,115]. Interestingly Pombo-Villar *et al.* [110] reported no such decrease in the case of the corresponding bis-sodium salt (entry 3). The reason for this apparent anomaly is unclear as, under assay conditions generation of the dicarboxylic acid would be expected. The data in table 5a suggests that it is therefore important that we consider not only the ability of these compounds to ring open as the sole determining ability to ability to inhibit PP2A, but other additional factors must also be in play. Almost all other modification of the anhydride moiety of cantharidin analogues results in little or no inhibition of PP1 and PP2A, table 5b shows selected exceptions to this rule.

7-OXO-BRIDGE MODIFICATIONS

Modification of the 7-oxo-bridgehead of cantharidin has received little attention, presumably a result of the rapid loss of protein phosphatase inhibition [113]. Interestingly there are no literature reports of the corresponding 7-S or 7-NH analogues. Although unpublished data from our own laboratory has shown that the 7-thia compound is

Table 5b. Effect of Other Modifications of the Anhydride Bridge on the Inhibition of PP21 and PP2A

			Protein Phosphatase Inhibition[c]		
Entry	Compound	R	PP1	PP2A	Ref.
1		-	-	608 nM	[109]
2		CH_3	4.71μM	0.41μM	[31]
3		CH_3CH_2	2.96μM	0.45μM	[31]
4		$CH_3CH_2CH_2$	4.82μM	0.47μM	[31]
5		CH_3	>1000μM	>1000μM	[31]
6		CH_3CH_2	746μM	55μM	[31]
7		$CH_3CH_2CH_2$	>1000μM	>1000μM	[31]

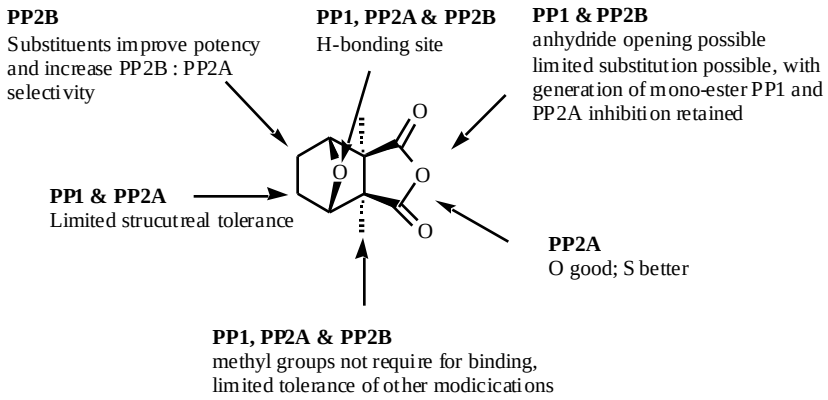


Fig. (3). Schematic representation of some of the important features required for inhibition of protein phosphatases 1, 2A and 2B. Note that no structure activity data is available for cantharidin analogues inhibiting PP2C.

completely inactive at PP1 and PP2A. It is possible that such modification, with the introduction of different H-bonding properties (cf 7-oxo) and the ability to synthetically modify the proposed 7-aza substituent, may retain or even enhance the inhibition of protein phosphatases.

MULTIPLE MODIFICATIONS

It is known that cantharidin (and tautomycin) bind in the active site of PP1 and PP2A in the ring-opened dicarboxylic acid form, however it has also been shown that in some instances that ring opening prior to binding actually results in a small, but significant decrease in inhibition [111]. Few compounds with multiple modifications retain their ability to inhibit protein phosphatases [75,111,112].

From our own original report we generated a basis model of important features for the inhibition of PP2A, this is shown in figure 3.

8. CONCLUSIONS

In conclusion, serine/threonine protein phosphatases are potential targets for therapeutic exploitation. However, the current array of protein phosphatase inhibitors falls short in many ways as being the ideal therapeutic agents. In this review we have focused on the development of small molecule inhibitors particularly those based upon the chemical structure of cantharidin. This agent not only represents the new generation of small molecule inhibitors but it also has a history of therapeutic use, particularly in the treatment of cancers. Unfortunately, it is only moderately selective for PP2A versus PP1, and it does possess some unwanted clinical side effects. In this regard agents based upon norcantharidin may well be more fruitful. In this short review we have shown how specific modifications of the cantharidin skeleton allows the development of specific protein phosphatase inhibitors. This work is still in its infancy and we believe that in the next few years more potent and selective inhibitors will be developed and introduced into the clinic.

ABBREVIATIONS:

PP	=	Protein phosphatase
I-1	=	Inhibitor-1
I-2	=	Inhibitor-2
DARPP-32	=	cAMP-Regulated phosphoprotein
NIPP-1	=	Nuclear inhibitor of protein phosphatase 1
PHAP-I	=	I-1PP2A, Putative histocompatability leukocyte antigens class II associated protein I
PHAP-II	=	I-2PP2A, SET, Putative histocompatability leukocyte antigens class II associated protein II
pRb	=	Retinoblastoma protein
cdk	=	Cyclin dependent kinases
CFTR	=	Cystic fibrosis transmembrane conductance regulator chloride channel
MAPK	=	Mitogen-activated protein kinases
MKK	=	MAPK Kinases

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