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CLASSIC PAPER

Sorting the protein phosphatases: okadaic acid led the way

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A seminal paper by Corinna Bialojan and Akira Takai [(1988) Biochem. J. **256**, 283–290] examined the effects of a compound extracted from marine black sponges, okadaic acid, on protein phosphatases. The identification of okadaic acid as a potent inhibitor of particular PPP family phosphatases was an extremely important milestone, not only in the differentiation of different protein phosphatases, but also in processes by which these protein phosphatases were identified as *in vivo* regulators of cellular signalling. In the present article, I review the information that led to the recognition of okadaic acid as a protein serine/threonine phosphatase inhibitor, and examine its currently known specificity and uses. I discuss its mode of inhibition and compare its interaction at the catalytic centre with those of more recently identified protein phosphatase inhibitors and inhibitory domains. Finally I consider the ongoing process of finding more specific protein phosphatase inhibitors.

Evolution has shaped a curious selection of naturally occurring inhibitors of phosphatases that dephosphorylate phospho-serine and -threonine residues in proteins. Their effects range from diarrhoetic food poisoning and lethality to tumour promotion, while their uses over centuries include wart removal and tumour suppression, aphrodisiac effects (of questionable efficacy!) and, more recently, immunosuppression that has changed the face of transplantation surgery (reviewed in [1,2]). The first of these rather surprising inhibitors was okadaic acid.

Akira Takai had been studying in Nagoya University at the time when research groups in Japan and the U.S.A. were isolating compounds from marine black sponges of the genus *Halichondria* (Figure 1). A complex molecule ($C_{44}H_{66}O_{13}$) was isolated from *H. okadai* by Dr Yasumasa Tsukitani at the Fugisawa Pharmaceutical Company in Tokyo and provided to Kazuo Tachibana, who determined the chemical structure [3]. At the time Kazuo Tachibana, now Professor in the Department of Bioorganic Chemistry, University of Tokyo, was then undertaking research for his doctorate thesis in the laboratory of the late Professor Paul J. Scheuer of Hawaii University. Paul Scheuer (1915–2004), a 'guru' of marine natural products chemistry, coined the name okadaic acid. As it transpired, okadaic acid is not made in sponges but is synthesized by marine dinoflagellates (marine plankton) and accumulates in marine organisms such as sponges and shellfish, which feed on them. The consequence to the human populations that eat shellfish with high levels of okadaic acid is rather unpleasant diarrhoetic seafood poisoning and the problem was unwelcome to the shellfish industry, but to the biochemist okadaic acid was a godsend!

Following studies from research groups in Nagoya, Tokyo and Honolulu which indicated that okadaic acid had potent effects on the contractility of intestinal smooth muscles [4] and the heart [5], Akira Takai, with an MD and doctorate in medical sciences at Nagoya University, became a research fellow of the Alexander-von-Humboldt Foundation in Heidelberg University. Shortly after his arrival, Akira met with Professor Caspar Rüegg, who spoke of "an interesting compound isolated from a Japanese black sponge, which had an enhancing effect on smooth muscle contraction", which he had learnt about from reading the recently published paper [4] and a manuscript under review [6]. At his suggestion, Akira wrote to Dr Tsukitani at Fugisawa Pharmaceutical Company in Tokyo and Yasumasa Tsukitani then very kindly sent 2.3 mg of okadaic acid - at current prices, worth ~£10,000 !

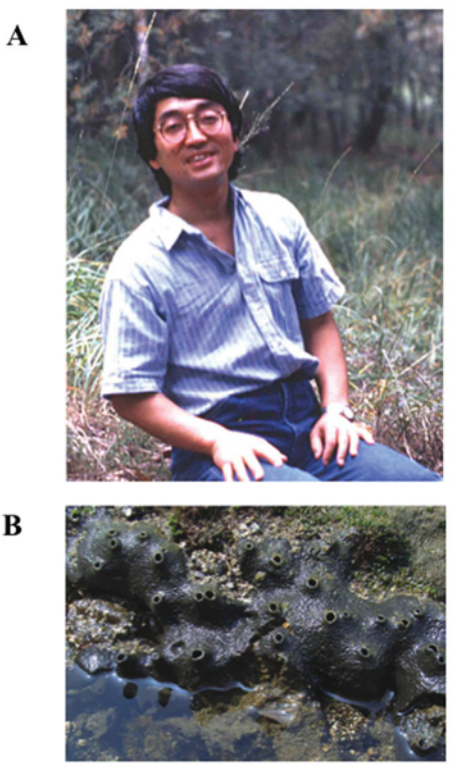

Figure 1 (A) Akira Takai in 1988, and (B) the marine black sponge *Halichondria okadai*

With this precious compound in hand, Akira Takai, Corinna Bialojan, Caspar Rüegg and colleagues examined the intracellular regulatory mechanisms known to activate muscle contraction via phosphorylation of myosin light chains [7,8]. They showed that okadaic acid did not affect the activity of the kinase that phosphorylates myosin light chains [MLCK (myosin light chain kinase)] but potently inhibited the phosphatase activity, which dephosphorylates myosin light chains. Since the level of protein phosphorylation is always a balance between kinase and phosphatase activities, myosin light chain phosphorylation was increased by okadaic acid, explaining the enhanced contractile

Figure 2 Corinna Bialojan in the late 1980s

response and, after the removal of Ca^{2+} ions, the slower relaxation. Furthermore, this effect on phosphatase activity was not limited to myosin phosphatase, because a purified aortic phosphatase [PCM (polycation modulated)] was similarly potently inhibited.

The 1988 *Biochemical Journal* article by Corinna Bialojan (then an assistant professor at Heidelberg University; Figure 2) and Akira Takai [9], which compared the effects of okadaic acid on a variety of protein phosphatases, has received at least 1275 citations. Bialojan and Takai [9] showed that PP1c (protein phosphatase 1 catalytic subunit) and PP2Ac were potently inhibited by okadaic acid in the nanomolar range, PP2B/calcineurin was inhibited in the micromolar range, whereas the Mn^{2+}/Mg^{2+}-dependent protein phosphatase (PP2Cα/PPM1A), phosphotyrosine phosphatases, inositol trisphosphate phosphatase, acid phosphatases and alkaline phosphatases were unaffected by up to 10 μM okadaic acid. Thus the studies identified a potent inhibitor that could distinguish the activities of two protein phosphatase catalytic subunits (PP1c and PP2Ac) from other phosphatase activities. At the time of publication, the protein phosphatases were difficult to identify in cell extracts, because their *in vitro* substrate specificities are very broad. The phosphatases that dephosphorylated serine and threonine residues in proteins were classified by four distinct types of catalytic subunit, PP1c, PP2Ac, PP2B/calcineurin-A and PP2C, based on their sensitivity to metal ions and the inhibitor-1 and inhibitor-2 proteins [10] that inhibited PP1c [11], but no molecules that inhibited PP2Ac were known. Okadaic acid inhibition differentiated PP1c and PP2Ac from PP2C and suggested that PP1c, PP2Ac and PP2B/calcineurin-A were more closely related. In the current classification of protein phosphatases, based on the amino acid sequences of the catalytic subunits (Tables 1 and 2), PP1c, PP2Ac and PP2B/calcineurin-A are members of the PPP family, whereas PP2C has a different amino acid sequence and is the founding member of the PPM family, which now has at least 17 members [12].

Okadaic acid inhibited PP2Ac activity with an IC_{50} of < 2 nM using two different substrates, phosphorylase *a* (phosphorylated with phosphorylase kinase) or myosin light chains (phosphorylated with MLCK). In contrast, the activity of PP1c on the

Table 1 Protein serine/threonine phosphatase families

The classification into three families is based on their amino acid sequences [12].

Abbreviation	Full name
PPP	Phosphoprotein phosphatases
PPM	Protein phosphatases, Mg^{2+}/Mn^{2+}-dependent (PP2Cα and 16 other related phosphatases)
FCP	Transcription factor IIF-interacting C-terminal domain phosphatase-related phosphatases

Table 2 The PPP family (catalytic subunits)

α, β/δ and γ are catalytic subunit isoforms. The terms PP1 (PP2A, PP2B) are sometimes used to denote the catalytic subunit as well as more generally to denote PP1 (PP2A, PP2B) complexes that may be of unknown or mixed composition. PP5/Ppp5 and PPEF/PP7/Ppp7 isoforms are monomeric. Note that purified preparations of PP1 (PP2A, PP2B) catalytic subunits from tissues are often a mixture of isoforms. Protein sequences can be located at http://www.uniprot.org/. No PPM or FCP family phosphatases tested show significant inhibition by the inhibitors listed below. c, catalytic subunit; CNA/CALNA, calcineurin A (catalytic) subunit.

Protein name	Human gene name	Activators	Inhibitors (nM)
PP1α/PP1cα/Ppp1cα PP1β/PP1δ/PP1cβ/Ppp1cβ PP1γ/PP1cγ/Ppp1cγ	*PPP1CA* *PPP1CB* *PPP1CC*		Inhibitor-1, inhibitor-2, okadaic acid, microcystin
PP2Aα/PP2Acα/Ppp2cα PP2Aβ/PP2Acβ/Ppp2cβ	*PPP2CA* *PPP2CB*		Okadaic acid, microcystin, fostriecin
PP4/PPX/PP4c/Ppp4c	*PPP4C*		Okadaic acid, microcystin, fostriecin
PP6/PP6c/Ppp6c	*PPP6C*		Okadaic acid, microcystin
PP2Bα/CNA/CALNA/Ppp3cα PP2Bβ/CNA2/CALNA2 CALNB/Ppp3cβ PP2Bγ/CNAγ/Ppp3cγ	*PPP3CA* *PPP3CB* *PPP3CC*	Ca^{2+}/calmodulin	FKBP12-FK506, cyclosporin-cyclophilin
PP5/PPT/Ppp5/Ppp5c	*PPP5C*	Arachidonic acid	Okadaic acid, microcystin
PPEF1/PP7α/Ppp7α/Ppp7cα PPEF2/PP7β/Ppp7β/Ppp7cβ	*PPP7CA** *PPP7CB**	Ca^{2+}/calmodulin	

*The gene names *PPP7CA* and *PPP7CB* are referred to as *PPEF1* and *PPEF2* in some databases.

Table 3 Inhibition of PPP catylic subunits by some naturally occurring inhibitors

The approximate IC_{50} is in nM unless otherwise stated. PP2Ac binds to okadaic acid, microcystin and calyculin A stoichiometrically, so the IC_{50} varies with the concentration of phosphatase in the assay. Note that the IC_{50} may also vary with the assay conditions and *in vivo* because most catalytic subunits in the PPP family form complexes with a large variety of interacting subunits. The inhibitors are membrane permeable but microcystin-LR is not readily taken up by most cell types. The IC_{50} is the concentration of inhibitor that causes 50 % inhibition of phosphatase activity. The data are derived from [19] and [21] and references therein. ND, not determined.

	Approximate IC_{50}						
Inhibitor	PP2Ac	Ppp4c	Ppp5	PP1cγ	PP2B/CN*	Ppp7	PP2C/Ppm1A
Okadaic acid	0.1	0.1	1.4	10	$\sim 4\ \mu$M	$> 1\ \mu$M	None
Microcystin-LR	0.1	0.1	1.0	0.2	$\sim 1\ \mu$M	$> 1\ \mu$M	None
Calyculin A	0.1	0.2	3	0.4	$> 1\ \mu$M	$> 1\ \mu$M	ND
Tautomycin	0.4–30	0.4	10	0.2–20†	$> 1\ \mu$M	ND	ND
Cantharidin	50	50	50	$> 1\ \mu$M	$> 10\ \mu$M	$> 20\ \mu$M	ND
Fostriecin	1.5	3	$> 50\ \mu$M	45 μM	$> 100\ \mu$M	ND	ND

*PP2B/CN requires the B regulatory subunit for assay of activity in the presence of Ca^{2+} and calmodulin.

†The IC_{50} of PP1cγ for tautomycin is not linear with the concentration of tautomycin so that with care, tautomycin can be used to inhibit PPC1γ more potently than PP2Ac in cells [1].

same two substrates was inhibited by okadaic acid with an IC_{50} of ~ 300 nM {with such potent inhibition, a significant fraction of inhibitor molecules become bound to the enzyme, causing non-linearity of Michaelis–Menton reciprocal plots at low substrate concentrations [13] and later analyses indicate the IC_{50} for okadaic acid with PP2Ac is 0.1 nM and with PP1c is 10 nM (Table 3)}. In contrast, the Ca^{2+}-stimulated calmodulin-dependent protein phosphatase PP2B/calcineurin was not inhibited in the nanomolar range, but exhibited an IC_{50} of 3–5 μM using phosphorylated myosin light chains or *p*-nitrophenolphosphate as substrates.

Although a very clear distinction between PP1 and type 2A phosphatase activities may be difficult to obtain with okadaic acid alone, the use of a combination of inhibitor-2 and okadaic acid can effectively distinguish them in cell lysates [2,14]. Okadaic acid rapidly traverses cell membranes, a property that has facilitated the identification of some of the substrates of PP1 and PP2A complexes in intact cells [15], although further methods are necessary to unambiguously differentiate between PP1 and PP2A substrates because inhibitor-2 does not cross the cell membrane. The recognition that the PPP family of phosphatases has more family members than were known in 1988 means that some studies identifying PP2A substrates employing okadaic acid need to be re-evaluated. Ppp4c and Ppp6c are closely related to PP2Ac, although they regulate distinct cellular functions, and Ppp4c, Ppp6c and Ppp5 (but not Ppp7/PPEF) are inhibited by okadaic acid with very similar IC_{50} values to that for PP2Ac (Table 3). Additional information is therefore needed to confirm which phosphatase is involved in a particular dephosphorylation or cellular process.

The inclusion of 100 nM okadaic acid to lysis buffers facilitated the identification of phospho-proteins dephosphorylated by PP1 and the type 2A phosphatase activities. In addition, the reversible nature of okadaic acid inhibition has been useful in the assay of the phosphatase activities of PP1 complexes, where the regulatory subunit is phosphorylated [16]. Thus the importance of the studies in the 1988 *Biochemical Journal* paper [9] was they provided the biochemist with an extremely useful tool to study cellular signalling and other processes regulated by serine/threonine phosphorylation.

Discoveries of other naturally occurring PPP inhibitors followed that of okadaic acid and have aided further differentiation of protein phosphatases [17] (Table 3), but none distinguish between PP2Ac and Ppp4c, and probably Ppp6c, for which information is not available. Fostriecin from *Streptomyces pulveraceus* (subspecies *fostreus*), discovered empirically by screening for anti-tumour agents, only inhibits PP1 and Ppp5 in the micromolar range but potently inhibits both Ppp4c and PP2Ac at low nanomolar concentrations [18,19]. Most notable was the recognition that cyclosporin A, which was known to have immunosuppressive properties, specifically bound to an intracellular protein cyclophilin A and the complex potently inhibited PP2B/calcineurin [20], a key component of the signalling pathway crucial for the activation of T-cells. Nevertheless, there are still some difficulties and caveats in assigning a function to a particular PPP family member [21].

So how do okadaic acid and other toxins inhibit type 2A-like PPs and PP1? The binding of okadaic acid close to the active site could be predicted by the mixed competitive and non-competitive inhibition demonstrated in the article by Bialojan and Takai [9]. More than 10 years later, the crystal structure of okadaic acid bound to protein phosphatase 1 showed that the toxin blocks substrate access and catalysis by interacting with the catalytic centre of PP1c and contacts the loop between β-strands 12 and 13 which overhangs the catalytic site, as well as binding to a hydrophobic groove [22,23]. Similar interactions have been noted with inhibitory molecules of varied structure. The interaction of PP1c with calyculin A, an inhibitor that possesses a phosphate group, is shown in Figure 3(A) [24]. The phosphate group (yellow) binds at the catalytic centre and the hydrophobic region of calyculin A binds to the hydrophobic groove of PP1c that normally associates with the backbone of protein substrates. Figure 3(B) shows the catalytic centre of PP1c bound to okadaic acid, superimposed on the catalytic centre of Ppp5; both catalytic sites are very similar to that of PP2Ac. In the absence of inhibitors, cleavage of a substrate–phosphate bond is initiated by a water molecule polarized by the metal ions, M1 and M2. However, Ppp5 possesses an autoinhibitory TPR domain with a glutamic acid (residue 76) that occupies a very similar position to that of the phosphate in calyculin A [25]. The carboxylate group of Ppp5 Glu^{76} in the inhibitory TPR domain and the phosphate group of calyculin A contact homologous arginine residues (Arg^{275} in Ppp5 and Arg^{96} in PP1c; Figure 3B). Okadaic acid bound to PP1c occupies a slightly different position from Ppp5 Glu^{76} and the phosphate of calyculin A, but the hydroxyl in the C2 position of okadaic acid nevertheless contacts Arg^{96} of PP1c, an amino acid that also interacts with the phosphate group of substrates. Figure 3(C) illustrates that a glutamic acid residue in microcystin, a toxin with an entirely different structure from okadaic acid and calyculin A, similarly contacts Arg^{96}. In all these cases, the inhibition of the PPP phosphatases involves binding of an

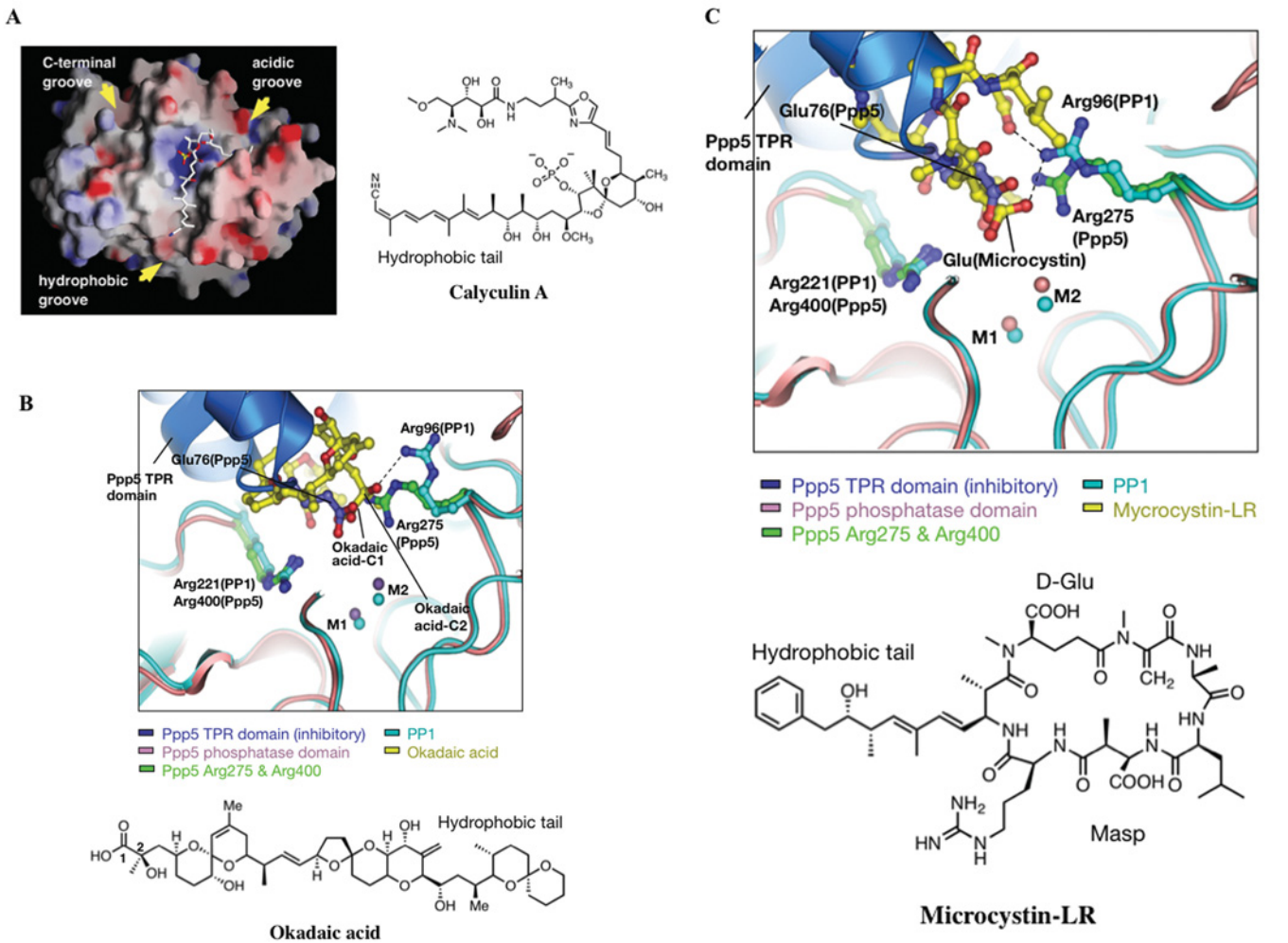

Figure 3 Interactions of the phosphatase inhibitors calyculin A, okadaic acid and microcystin-LR, with PP1c: comparison with those of the inhibitory domain of Ppp5 at the catalytic centre of Ppp5

(**A**) The molecular surface of the PP1cγ–calyculin A complex {reproduced from Figure 3 in [24] with kind permission from the authors and © (2002) Elsevier}. Negatively charged surfaces of PP1cγ are in red, and positively charged surfaces are in blue. The charges on the metal ions at the catalytic centre were assigned +2. The three main grooves on PP1cγ are indicated by arrows. Calyculin A is shown in white sticks with the phosphate group in yellow. The chemical structure of calyculin A is shown on the right. (**B**) Superimposition of the catalytic centre of PP1cγ complex with okadaic acid on the autoinhibited catalytic centre of Ppp5. The contact of the C2 hydroxyl of okadaic acid with Arg^{96} is indicated (derived from the crystal structure in [22]). Arg^{96} and Arg^{221} are predicted to interact with the phosphate group of substrates [26]. M1 and M2 are metal ions (probably iron and zinc in the endogenous enzymes) at the catalytic centre. Ppp5 possesses an autoinhibitory tetratricopeptide repeat (TPR) domain, with the carboxylate group of Glu^{76} contacting Arg^{275} (green) of the phosphatase domain of Ppp5, which is homologous to Arg^{96} in PP1cγ [25]. Calyculin A (not shown) contacts Arg^{96} and Arg^{221} of PP1cγ. The chemical structure of okadaic acid is shown below. (**C**) Superimposition of the catalytic centre of PP1cα complex with microcystin-LR [32] on autoinhibited catalytic centre of Ppp5. The α-carboxylate group of γ-linked D-glutamic acid in microcystin-LR contacts PP1cα Arg^{96} and is in a virtually identical position with the carboxylate group of Glu^{76} of Ppp5 [25]. The contact of the carboxylate group of Masp (D-*erythro*-β-methyl aspartic acid) with PP1cα Arg^{96} is also indicated. The chemical structure of microcystin-LR is shown below. Figures 3(B) and 3(C) were based on Figure 4 in [25] and kindly provided by David Barford. Adapted with permission from Macmillan Publishers Ltd: *EMBO Journal* [25], copyright (2005). (www.nature.com/emboj/index.html)

anionic group of the toxin or inhibitory domain at the catalytic site [25], the mode of interaction partially mimicking that of tungstate (an analogue of phosphate) in the PP1c structure [26]. One might expect that changes in the okadaic acid interaction at the phosphatase catalytic centre would account for the more potent inhibition of PP2Ac compared with PP1c, but recent data does not show any major differences between the catalytic centre of the PP2Ac–okadaic acid complex from that observed for PP1c–okadaic acid in Figure 3(B). Instead, resolution of the crystal structure of PP2Ac in complex with okadaic acid revealed that the hydrophobic end of okadaic acid, which binds to the hydrophobic groove, is held in place by a hydrophobic cage in PP2Ac that is absent in PP1c [27]. It is suggested that replacement of amino acids Ser^{129}, Ile^{130}, Asp^{197} and Trp^{206} in PP1c by a different set (Gln^{122}, Ile^{123}, His^{191} and Trp^{200}), which form the hydrophobic cage in PP2Ac, underlies the increased affinity of PP2Ac for okadaic acid. However, mutations in the β12–13 loop (^{268}SAPNYCGEFD^{277} in PP1c) also modify the affinity of the PPPs for toxins [1]. In particular, replacement of Phe^{276} in PP1c by cysteine (the residue present in PP2Ac) increased the affinity for okadaic acid [28]. This region may also contribute to the lower affinity of PP2B/calcineurin for okadaic acid [29].

In 1988, all protein phosphatases tested with okadaic acid were endogenous forms, most kindly donated by a variety of groups. In order to express PP1c in bacteria for crystallographic studies in complex with inhibitors, Akira came to Dundee in March 1997 and with the help of a postdoctoral scientist, Hazel Barker (in my research group), he generated an excellent PP1cγ

preparation. But perhaps the most memorable aspect of the visit was the charming flute duet he played with my young daughter. A recent recording of Akira playing the first movement of Bach's Sonata in B minor, accompanied by his wife and ophthalmologist, Yoshiko (née 'Okada'!) can be heard at http://www.asahikawa-med.ac.jp/dept/mc/phys1/files/music/BWV1030-1.mp3

Okadaic acid has been described as a polyether derivative of a C_{38} fatty acid, and this complex molecule of 805 Da was chemically synthesized soon after it was identified [30]. However, synthesis is by no means straightforward [31] and most forms of okadaic acid marketed are still extracted from marine organisms. So is okadaic acid a tumour promoter or tumour suppressor? Well, that depends not only on which PPP catalytic subunit (PP1c, PP2Ac, Ppp4c, Ppp5 or Ppp6c) is being inhibited, but on which complexes of these phosphatases, since all except Ppp5 interact with a plethora of regulatory subunits. Whether okadaic acid functions to promote or inhibit tumour growth therefore depends on the concentration, duration of treatment and type of cell or tissue being treated. Identification of small molecule ($\leqslant$ 300 Da) inhibitors of the PPP family members has proved difficult. FK506 is the only synthetic small molecule inhibitor identified and, similar to cyclosporin, it forms a complex with an intracellular cyclophilin (FKBP12) and inhibits PP2B/calcineurin. Inhibitors that are specific for Ppp4c, Ppp5 and Ppp6c and distinguish them from PP2Ac will be a bonus to the biochemist and one may even provide an anti-cancer agent.

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