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Fostriecin, an antitumor antibiotic with inhibitory activity against serine/threonine protein phosphatases types 1 (PP1) and 2A (PP2A), is highly selective for PP2A

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Abstract Fostriecin, an antitumor antibiotic produced by *Streptomyces pulveraceus*, is a strong inhibitor of type 2A (PP2A; IC_{50} 3.2 nM) and a weak inhibitor of type 1 (PP1; IC_{50} 131 μM) serine/threonine protein phosphatases. Fostriecin has no apparent effect on the activity of PP2B, and dose-inhibition studies conducted with whole cell homogenates indicate that fostriecin also inhibits the native forms of PP1 and PP2A. Studies with recombinant PP1/PP2A chimeras indicate that okadaic acid and fostriecin have different binding sites.

Key words: Fostriecin; Okadaic acid; Cantharidin; Serine/threonine; Protein; Phosphatase; Inhibitor; Dephosphorylation; Toxin; Tumor promotion; Antitumor activity

1. Introduction

Fostriecin (Fig. 1) is an antitumor antibiotic isolated from the fermentation beer of *Streptomyces pulveraceus* (subspecies *fostreus*) [1,2] that is under development as an anticancer agent [3]. Fostriecin exhibits antitumor activity against a wide spectrum of tumor cells in vitro and has excellent activity against L1210 and P388 leukemias in vivo [4,5]. Currently, fostriecin is under evaluation as an antitumor drug in clinical trials.

The cellular mechanisms underlying the antitumor activity of fostriecin have been under investigation for several years. Early studies indicated that fostriecin rapidly inhibits DNA, RNA and protein synthesis [6]. Although these studies demonstrated that fostriecin affects a process essential for replication and transcription, no direct effect on DNA polymerase, RNA polymerase or enzymes within the purine or pyrimidine biosynthetic pathways was noted [3,6]. Subsequently, fostriecin was shown to block cells in the G2 phase of the cell cycle and to have inhibitory effects on partially purified type II topoisomerase from Ehrlich ascites carcinoma [7]. However, fostriecin is distinctly different from previously described inhibitors of topoisomerase II in that it does not cause protein-associated DNA strand breaks [7].

Recent studies suggest that the antitumor activity of fostriecin may originate from its ability to interfere with the reversible phosphorylation of proteins that are critical for progression through the cell cycle [8–10]. Fostriecin has been reported to inhibit a mitotic entry checkpoint [8], and it induces chromosome condensation in interphase cells in a manner comparable to okadaic acid, a known inhibitor of protein phosphatases types 1 (PP1) and 2A (PP2A) [9]. Fostriecin induced chromosome condensation correlates with an increase in histone H2A and H3 phosphorylation in mouse mammary tumor cells, and fostriecin induces the hyperphosphorylation of vimentin, which correlates with the reorganization of intermediate filaments in hamster kidney cells [10]. Furthermore, fostriecin inhibits the dephosphorylation of a phosphopeptide (KRphospho-T-IRR; Upstate Biotechnology, Inc.) by partially purified PP1 and PP2A [8].

Other inhibitors of PP1 and PP2A, notably okadaic acid [11,12] and microcystin-LR [13,14], have been reported to have tumor promoting activity [15,16], while cantharidin [17,18] and structural derivatives of cantharidin, have antitumor activity against KB cells in culture and on reticulocell sarcoma and ascites hepatoma in mice [19,20]. Therefore, if the antitumor activity of fostriecin results from the inhibition of PPase activity, the further characterization of the inhibitory effects of fostriecin on specific PPase is clearly warranted. The present study characterizes the inhibitory activity of fostriecin employing the purified catalytic subunits of serine/threonine protein phosphatases types 1 (PP1), 2A (PP2A) and 2B (PP2B). This study also characterizes the effects of fostriecin on native PPases contained whole cell homogenates of the well characterized Rin5mF insulinoma cells [21,22]. These studies indicate that fostriecin is a $>10\,000$-fold more potent inhibitor of PP2A than PP1 and has no apparent effect on PP2B. Furthermore, the inhibition of PPase activity with both the purified PPase and the dilute cell homogenate correlates well with the concentrations that have antitumor activity in vitro and in vivo [1–4], adding additional support to the concept that the antitumor activity of fostriecin is associated with the inhibition of PPase activity.

2. Materials and methods

2.1. Materials

Phosphorylase kinase (EC 2.7.1.38) from rabbit muscle, PP2B (calcineurin) and calmodulin (phosphodiesterase 3′,5′-cyclic nucleotide activator) from bovine brain, and *p*-nitrophenyl phosphate (PNPP) were obtained from Sigma Company and utilized without further purification. Fostriecin (NSC 339638, lot 700-95-202) was generously donated by the Division of Cancer Treatment, National Cancer Institute and Parke-Davis. Okadaic acid was generously provided by Dr. R. Dickey.

2.2. Preparations of phosphoprotein substrates

[^{32}P]Phosphorylase *a* from rabbit muscle was prepared essentially according to the methods of Brautigan and Shriner [23] and described previously [13,24,27]. Briefly, 20 mg of phosphorylase *b* was incubated with 1.4 mCi of [γ-^{32}P]ATP (1×10^4 cpm/pmol) and 100 units of phosphorylase kinase for 1.5 h at pH 8.2 and 30°C. After the phosphorylation reaction was terminated, phosphorylase *a* was crystallized

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by adjustment of the pH to 6.8 and placing the mixture on ice. The crystals were collected by centrifugation and washed extensively with ice cold 20 mM Tris-HCl, 50 mM 2-mercaptoethanol, pH 6.8 (buffer A). After washing, the crystals were dissolved by the addition of 100 mM NaCl and heating to 30°C. This solution was then passed through a Sephadex G-25 column and the protein containing fraction was dialyzed against buffer A [24]. This procedure produces phosphorylase *a* with a specific activity of $\sim 5.8 \times 10^6$ cpm/nmol of incorporated phosphate.

2.3. Determination of protein phosphatase activity

The catalytic subunit of PP1 was purified to apparent homogeneity, demonstrating a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, essentially according to the methods of Brautigan et al. [26] and described in Honkanen et al. [17,27,28]. The catalytic subunit of PP2A was purified to apparent homogeneity as described by Pallas et al. [29], using G-75 Sephadex in the place of Ultrogel-AcA44 as previously reported [24–28].

Protein phosphatase activity against phosphorylase *a* was determined by the liberation of ^{32}P using either the purified catalytic subunits of the indicated protein phosphatases (Fig. 2) or a dilute whole cell homogenate of RIN5mF insulinoma cells (Fig. 3). Assays (80 μl total volume) containing 50 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 1 mM EDTA and [^{32}P]phosphorylase *a* (1–2 μM PO_4) were conducted as described by Honkanen et al. [17,24]. For all reactions the dephosphorylation of substrate was $< 10\%$ of the total, and reactions were linear with respect to enzyme concentration and time. [^{32}P]Phosphate liberated by the enzymes was extracted as a phosphomolybdate complex and measured according to the methods of Killilea et al. [30]. Inhibition of phosphatase activity was determined by the addition of fostriecin or okadaic acid to the assay mixture 10 min before initiation of the reaction with the addition of substrate [24]. Fostriecin was dissolved in phosphate buffered saline containing 0.1 mM ascorbic acid (as an antioxidant), which was added in equal amounts to controls. Unless indicated otherwise, inhibition assays, conducted with purified rabbit muscle PP1 or PP2A, contained 200 pM PP1 or 225 pM PP2A, having phosphatase activity against phosphorylase *a* of 4.2 ± 0.25 and 1.1 ± 0.22 μmol min^{-1} mg of $protein^{-1}$, respectively. The phosphorylase *a* phosphatase activity of recombinant human PP1α and the PP1/PP2A chimera (CRHM2) was 1.1 ± 0.1 and 0.95 ± 0.09 μmol min^{-1} mg of $protein^{-1}$, respectively. For comparison studies conducted with CRHM2, PP2A and recombinant PP1 (Fig. 5), the amount of each PPase was adjusted so that equal amounts (~ 500 pM) of each PPase were contained in the assay. For kinetic analysis, 1–30 μM phosphorylase *a* (final concentration) was used as substrate. K_m and V_{max} values were estimated from Lineweaver-Burke plots.

PP2B activity was measured using (PNPP) in the presence of Ca^{2+} and calmodulin as described previously [17,24]. Briefly, the assay (180 μl total volume) containing 50 mM Tris base, pH 8.5, 20 mM $MgCl_2$, 0.25 mM DTT, 0.2 μM calmodulin, 0.1 mM $CaCl_2$, and 20 mM PNPP, was conducted at 30°C for 15 min. The reaction was terminated by the addition of 900 μl of 1 M $NaCO_3$, pH 10, and change in absorbance, measured at 410 nm, was used to calculate phosphatase activity.

2.4. Construction of PP1/PP2A chimeras

A clone containing the full-length PP2A cDNA from bovine brain was generously provided by Shu-Chih Chen (Pacific Northwest Research Foundation). PCR was used to amplify the entire coding region of PP2A, and the PCR product was subcloned into pBluescript (Stratagene). Recombinant human PP1α was obtained by PCR amplification of human retina cDNA using PP1α specific primers (RH18; 5′-CTGGACGAATTCATGTCCGACAGCGAG-3′ and RH17; 5′-TTATTCAAGCTTCCAGATGGGTTGCC-3′). The PCR product produced was subcloned into pBluescript and sequenced. The 3′-coding region (bp 796–933) of PP2A was then substituted for the 3′-coding region of PP1 (816–1021) using the gene fusion techniques described by S. Barik [31]. Briefly, a 30 base oligonucleotide containing 15 bases complementary to PP1 (bp 803–816) and 15 bases of PP2A (bp 796–811) was synthesized. This PP1/PP2A primer was used in combination with a PP2A specific antisense primer (SB8; 5′-GGGCCGGATCCATCAC AGGAAGTAGTC-3′) to amplify the C-terminal region of PP2A (bp 796–933). The PCR product produced was then employed as a 'mega-primer' in combination with a PP1 specific primer (RH18) to amplify PP1α cDNA. After 8 cycles, an aliquot of the PCR reaction was diluted and used as template for 30 additional rounds of amplification using an N-terminal PP1 specific sense (RH18) primer in combination with a PP2A C-terminal specific antisense (SB8) primer. The PCR product produced was then subcloned into pBluescript and sequenced to ensure the fidelity of the PP1/PP2A-chimera (CRHM2).

2.5. Expression of recombinant PPases

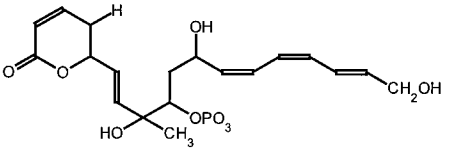
The coding region of PP1α and the chimera, CRHM2, were subcloned behind the *tac* promoter in pKK223-2 (Pharmacia) and used to transform a competent strain of *E. coli* (TOP 10 or JM105). For expression, the bacteria were grown in LB-amp supplemented with 1 mM $MnCl_2$ and 50 μM IPTG at 18°C for ~ 72 h. The bacteria were then collected by centrifugation and lysed with a French press. The recombinant PPases were purified employing essentially the same procedures used to purify the native catalytic subunits of PP1 and PP2A from animal tissue, omitting the methanol precipitation step prior to chromatography on heparin-Sepharose; like PP1, CRHM2 is retained on Heparin-Sepharose and elutes upon the addition of buffer containing 1 M NaCl (Walsh and Honkanen, unpublished observation).

3. Results

The dephosphorylation of phosphorylase *a* by the purified catalytic subunit of PP1 or PP2A is inhibited by fostriecin, which has no apparent effect on the activity of PP2B at the concentrations tested (Fig. 2). When diluted below the titration endpoint, defined as the concentration of enzyme after which further dilution no longer affects the IC_{50} [13,24], fostriecin inhibits the activity of both PP1 and PP2A in a dose-dependent manner, inhibiting PP2A ($IC_{50} = 3.2 \pm 0.2$ nM; mean ± S.D., $n = 4$) at a $\sim 40\,000$ fold lower concentration than PP1 ($IC_{50} = 131 \pm 13.1$ μM; mean ± S.D., $n = 4$).

To determine whether fostriecin inhibits the activity of the native forms of PP1 and PP2A at a comparable concentration to that observed with the respective purified catalytic subunits, we examined the effects of fostriecin in dilute (65 μg protein/ml) whole cell homogenates of RINm5F insulinoma cells (Fig. 3). Fostriecin inhibits the divalent cation-independent protein phosphatase activity in the whole-cell homogenate in a dose-dependent manner, with complete inhibition requiring the addition of ~ 10 mM fostriecin. Nonlinear regression analyses of the dose-inhibition curve obtained with fostriecin indicates that the divalent cation-independent phosphatase activity contained in the RINm5F cell homogenate best fits a multi-site model, which is consistent with that predicted for an assay containing a mixture of at least PP1 and PP2A [13,17,24].

To further characterize the interaction of fostriecin with PP1 and PP2A, a series of PP1/PP2A chimeras was constructed. The goal was to identify the fostriecin binding domain by producing mutations in PP1/PP2A displaying altered sensitivity to fostriecin. Unfortunately, most of the constructs had markedly decreased activity against phosphorylase *a* or altered kinetic properties (i.e. increased K_m and decreased V_{max}). This suggests that the mutations produce conforma-



Fostriecin

Fig. 1. Structure of fostriecin.

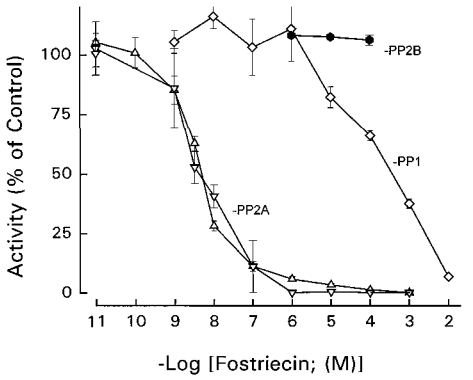


Fig. 2. Inhibition of protein phosphatases by fostriecin. The purified catalytic subunits of PP1 (◇) and PP2A (△,▽) were assayed, using [^{32}P]labeled phosphorylase *a* as a substrate, and PP2B (●) using PNPP as described in Section 2. The data are expressed as % of controls, with control activity against phosphorylase *a* being 4.2±0.25 and 1.1±0.22 μmol min^{-1} mg of protein^{-1} for PP1 and PP2A, respectively. Fostriecin was mixed with the enzymes for 10 min at 23°C prior to the initiation of the reaction with the addition of substrate. Inhibition assays contained ~200 pM PP1, 200 pM PP2A (△) or 100 pM PP2A (▽). The data represent the mean±S.D. (n=8) of data obtained from at least three separate experiments employing two different preparations of each enzyme tested.

tional changes in the enzyme, which makes it difficult to assess if an observed change in sensitivity to fostriecin results from a mutation occurring directly in the inhibitor binding domain or to the masking-unmasking of the binding domain subsequent to a conformational change in the enzyme. Nonetheless, one construct, CHRM2, was found to have kinetic properties (K_m 10.2 μM, V_{max} 48 μmol mg^{-1} min^{-1}) comparable to that of recombinant human PP1α (K_m 10.4, V_{max} 49.3 μmol mg^{-1} min^{-1}). In CRHM2, the COOH-terminal domain of PP1 (816–1021) is replaced by the COOH-terminal region of PP2A (bp 796–933), with the fusion occurring at a domain (SAPNYC) conserved in both PP1 and PP2A. As seen in Fig. 4, both endogenous PP2A and CRHM2 are potently inhibited by okadaic acid. However, when the concentration of either

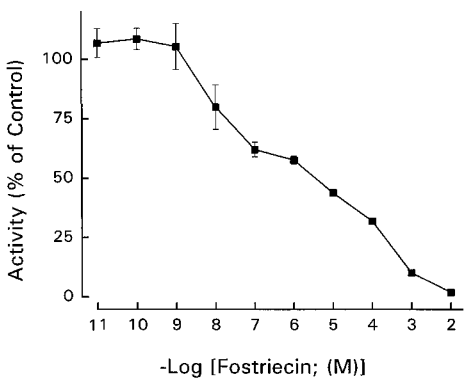


Fig. 3. Effect of fostriecin on protein phosphatase activity contained in a homogenate of RINm5F insulinoma cells. Cells from a single ~50% confluent 100 mm dish were rinsed with 4°C phosphate buffered saline, scraped and sonicated in 1 ml of Tris buffer (20 mM Tris-HCl, 1 mM EDTA, and 2 mM dithiothreitol, pH 7.4). The homogenate was then diluted below the titration endpoint (the concentration after which further dilution no longer affects the IC_{50} [13,24], ~65 μg protein/ml) and assayed for phosphorylase *a* phosphatase activity as described in Fig. 2. Each point represents the mean±S.D. of three experiments conducted in triplicate.

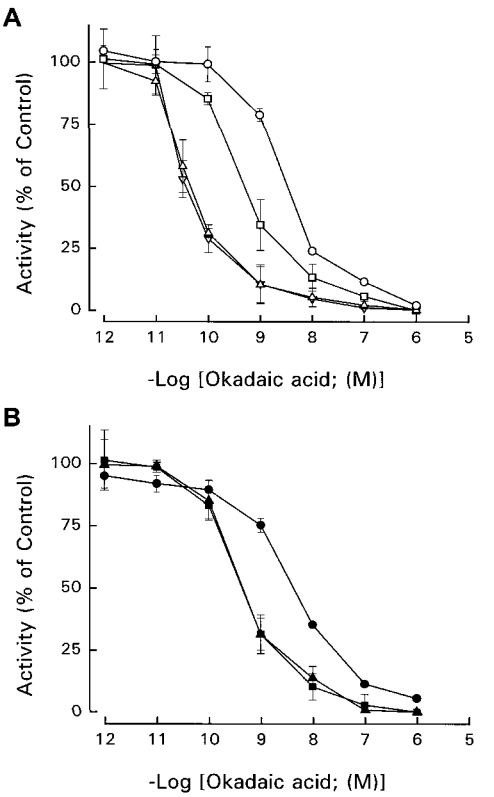


Fig. 4. Effect of enzyme concentration on the inhibition of purified catalytic subunits of PP2A and a recombinant PP1/PP2A chimera (CRHM2). Phosphatase activity was determined with phosphorylase *a* as a substrate (see Section 2 for details), and assays were conducted as described in Fig. 2, with the concentration of PP2A added to the assay adjusted to 5 nM (○), 500 pM (□), 200 pM (△) and 100 pM (▽) and the concertina of CRHM2 adjusted to 5 nM (●) 500 pM (■) and 200 pM (▲). Okadaic acid was mixed with each enzyme 10 min at 23°C prior to the addition of substrate. Each point represents the mean±S.D. (n=3).

PPase exceeds that of okadaic acid, the apparent IC_{50} of okadaic decrease with dilution (Fig. 4A,B). This 'shift in IC_{50}' appears to result from the titration of okadaic acid from the assay by the catalytic subunit of PP2A or CHRM2, because it is not observed in assays containing very low concentrations of enzymes and is also observed with microcystin-LR [13] and nodularin [24].

Once diluted below the titration endpoint, okadaic acid inhibits the activity of native PP2A and CRHM2 in a dose-dependent manner, inhibiting the activity of PP2A at a slightly lower concentration than CRHM2. This difference could indicate that part of the high affinity okadaic acid binding domain contained in PP2A has been replaced by a region of PP1 that renders CRHM2 less sensitive to okadaic acid. However, when compared to endogenous PP2A, CRHM2 is less active against phosphorylase *a*. Thus, the difference in sensitivity to okadaic acid could also result from a titration artifact produced by the presence of aberrantly folded recombinant CRHM2 that is inactive yet still capable of binding okadaic acid. Nonetheless, when the inhibitory effect of fostriecin is compared in assays containing equal amounts of endogenous PP2A and CRHM2 (effectively neutralizing any potential titration effects) CRHM2 is inhibited by okadaic acid at concentrations comparable to that of PP2A (Fig. 5). This is consistent with studies indicating that the okadaic acid

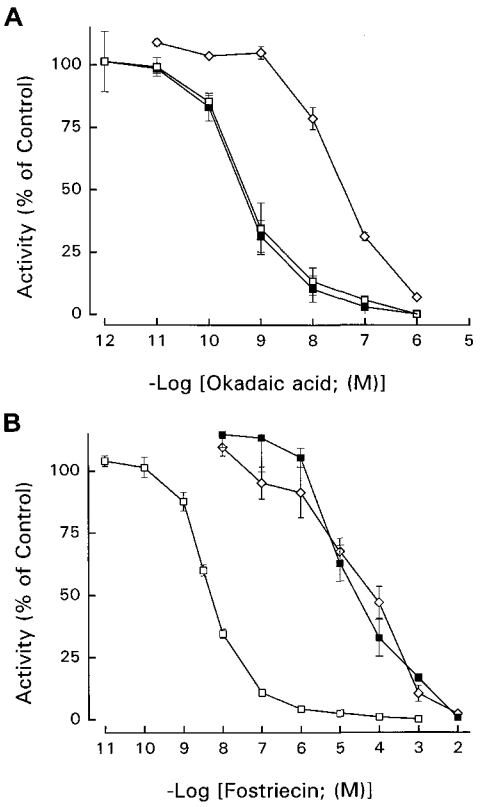


Fig. 5. Comparison of the inhibitory effect of okadaic acid and fostriecin on the activity of the catalytic subunit of PP2A (□), recombinant PP1α (◇) and a recombinant CRHM2 (■). Assays were conducted as described in Fig. 2 with all three enzymes added in comparable amounts (~500 pM).

binding site is contained in the C-terminal region of PP2A or a combination of the C-terminal region of PP2A and other regions conserved in both PP1 and PP2A [32–35]. In contrast, CRHM2 is inhibited by fostriecin at concentrations similar to those which inhibit PP1α, suggesting that the high affinity fostriecin binding domain is located in a different region of PP2A.

4. Discussion

To date, several potent inhibitors of PP1 and PP2A have been identified, including: okadaic acid, a polyether carboxylic acid produced by several species of marine dinoflagellates [11,12]; >50 variants of microcystin, a family of cyclic heptapeptides produced by certain strains of cyanobacteria [13,36,37]; nodularin, a cyclic pentapeptide produced by *Nodularia spumigena* [24]; motuporin, a structural variant of nodularin [34]; calyculin A, a spiro ketal obtained from a marine sponge [39]; tautomycin, a dialkylmaleic anhydride produced by a strain of *Streptomyces spiroverticillatus* [40–42]; and cantharidin, an oxabicycloheptane-2,3-dicarboxylic acid anhydride that is a natural toxicant of blister beetles [17,18]. This study confirms that fostriecin has inhibitory activity against PP2A and PP1, and shows that fostriecin has no apparent effect on the Ca^{2+}/calmodulin-dependent phosphatase, PP2B. In addition, fostriecin inhibits the divalent cation-independent PPase activity contained in dilute cell homogenates of RINm5F insulinoma cells at concentrations comparable to that which affect the purified catalytic subunits (Fig. 3). Thus, fostriecin represents yet another example of a natural compound with inhibitory activity against both the native and purified catalytic subunits of PP1 and PP2A. Furthermore, fostriecin is relatively water soluble and utilizes the reduced folate carrier system to enter cells [43,45]. This allows for the efficient delivery and uptake of fostriecin by many types of cultured cells (Walsh and Honkanen, unpublished observation), suggesting that fostriecin will also prove useful as a research tool.

Due to its ability to inhibit PP2A at 10–100-fold lower concentrations than PP1, okadaic acid has proven valuable as a research tool for determining the physiological and pathological roles of these serine/threonine protein phosphatases [12,44]. Like okadaic acid, fostriecin is a more potent inhibitor of PP2A than PP1, and the purified catalytic subunit of PP2A from muscle is inhibited by fostriecin at a concentration that is ~40 000 times lower than that required to inhibit the activity of purified PP1 (Fig. 2). Under identical assay conditions okadaic acid is more potent inhibitor of both PP2A and PP1 (Fig. 4C) [11,12,24,28]. However, the greater selectivity of fostriecin for PP2A should prove beneficial for distinguishing the activity of PP1 and PP2A in dilute cell homogenates, and the reduced potency of fostriecin may also diminish the potential for titration artifacts that often complicate the use of okadaic acid in studies designed to distinguish the activity of PP2A from that of PP1. In addition, the increased selectivity of fostriecin for PP2A may be useful in studies designed to clarify the paradox where inhibitors of the same PPases can act as antitumor antibiotics or tumor promoting agents [3,11–20]. Still fostriecin is sensitive to oxidation [38], rendering it less stable than okadaic acid (Walsh and Honkanen, unpublished observation).

The recent elucidation of the x-ray crystal structure of the catalytic subunit of PP1 complexed with microcystin-LR indicates that the COOH-terminal subdomain sits on the NH_2-terminal subdomain, forming three surface grooves (a hydrophobic groove, an acidic groove and a COOH-terminal groove), with the active site situated at the bifurcation point of the three grooves [35,46]. Microcystin-LR covalently binds to Cys-273 in PP1, which is adjacent to the predicted active site, and several studies predict that microcystin inhibits PP1 activity by blocking access to or interacting with key residues in the active site [34,35,46]. Modeling studies based on the crystal structures of free okadaic acid suggest that okadaic acid binds to the same region as microcystin [34], and mutagenesis analyses of PP1 suggest an important role of the β12/β13 loop (residues 272–276) in the inhibition of PP1 and PP2A by microcystin-LR, okadaic acid and calyculin A [32–35,46]. The inhibition studies conducted with CRHM2, where the C-terminal region of PP1 is replaced with the C-terminal region of PP2A, revealed that the recombinant PPase has increased sensitivity to okadaic acid. These findings are consistent with the findings of Zhang et al. [32,33] and Huang et al. [35], providing further support for the importance of the β12/β13 loop and adjacent regions for the binding of okadaic acid and microcystin [32–35,46]. In contrast to the effects observed with okadaic acid, CRHM2 was inhibited by fostriecin at concentrations similar to those needed to inhibit the activity of PP1. Thus, the high affinity fostriecin binding site of PP2A does not appear to be located in the same region as the okadaic acid and microcystin binding domain. The eluci-

dation of the crystal structures of PP2A and fostriecin should certainly aid in further defining the fostriecin binding domain.

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