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Inhibition of protein phosphatases-1 and -2A with acanthifolicin

Comparison with diarrhetic shellfish toxins and identification of a region on okadaic acid important for phosphatase inhibition

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Acanthifolicin (9,10-epithio-okadaic acid from *Pandoras acanthifolium*) inhibited protein phosphatase-1 (PP1) similarly to okadaic acid (IC_{50} = 20 nM and 19 nM, respectively) but was slightly less active against protein phosphatase-2A (PP2A) (IC_{50} = 1 nM and 0.2 nM, respectively). Methyl esterification of acanthifolicin sharply reduced its activity. PP2A was inhibited with an IC_{50} = 5.0 μM, whilst PP1 was inhibited < 10% at 250 μM toxin. Okadaic acid methyl ester was similarly inactive whereas dinophysistoxin-1 (35-methyl okadaic acid) inhibited PP1/2A almost as potently as okadaic acid. Pure acanthifolicin/okadaic acid methyl ester may be useful as specific inhibitors of PP2A at 1-10 μM concentrations in vitro and perhaps in vivo. The data also indicate that a region on these toxins important for PP1/2A inhibition comprises the single carboxyl group.

Protein phosphatase; Acanthifolicin; Diarrhetic shellfish toxin; Okadaic acid; Tumor promoter; Protein phosphorylation

1. INTRODUCTION

Okadaic acid (Fig. 1) is a complex polyether produced by marine dinoflagellates that accumulates in marine sponges such as *Halichondria okadaii* and in the digestive glands of shellfish [1,2]. Its structure is similar to acanthifolicin (Fig. 1), the first polyether carboxylic acid to be reported from marine sources [3]. Okadaic acid is a major cause of diarrhetic shellfish poisoning (DSP) and a potent non-phorbol ester type tumour promoter [4,5]. Its effects on smooth muscle contractility [6] led Takai et al. [6] to deduce that it was an inhibitor of protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A); two of the 4 major protein phosphatases in the cytosol of mammalian cells that dephosphorylate serine and threonine residues (reviewed in [8,9]). These and subsequent studies [10] established okadaic acid as a potent and specific inhibitor of PP1 (IC_{50} = 10–20 nM) and PP2A (IC_{50} = 0.1–0.2 nM). In order to begin to delineate in molecular terms the nature of protein-ligand interactions underpinning the inhibition of PP1 and PP2A by okadaic acid, we investigated the effects of synthetic and naturally occurring analogues of this toxin on these two enzymes.

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2. MATERIALS AND METHODS

2.1. *Sources of marine toxins*

Okadaic acid was obtained from Moana Bioproducts Inc., Harding Ave., Suite 304, Honolulu, HI, 9681, USA. The methyl ester of okadaic acid (methyl okadaate) was prepared and characterized as in [1]. Acanthifolicin was isolated from the sponge *Pandoras acanthifolium* and its methyl ester prepared as in [3]. Proton nuclear magnetic resonance data for this ester were: ^1H NMR ($CDCl_3$) 300 MHz (chemical shift (multiplet, J in H_z, proton assignments)); 0.93 (d, 6.75), 0.97 (d, 6.75, H-42), 1.06 (d, 6.75), 1.38 (s, H-44), 1.73 (s, H-43), 3.25–3.75 (7H, m), 3.80 (s, OCH_3), 3.90–4.20 (4H, m), 4.46 (q, 8, H-16), 5.07 (s, H-41), 5.38–5.47 (3H, m, H-14, 15, 41); exchangeable absorptions at 2.52, 3.01, 5.04. Besides these absorptions the spectrum contained complex overlapping multiplets in the range 1.20–2.35 ppm. The purity of the methyl esters was further checked by reverse phase liquid chromatography since these compounds could be resolved from their non-esterified counterparts. Detection by protein phosphatase bioassay indicated that acanthifolicin methyl ester was entirely free of acanthifolicin. Methyl okadaate contained 0.02% residual okadaic acid and inhibition data (Figs 2 and 3) were corrected to account for this contamination.

9-Anthryldiazomethyl (ADAM) ester of okadaic acid was a generous gift from Dr J. Marr, Oceanchem. Group, Dartmouth, Nova Scotia and dinophysistoxin-1 (35-methyl okadaic acid, DTX-1) was obtained from Dr M. Quilliam, NRC, Halifax. Monensin and nigericin were from Sigma, salinomycin from Calbiochem and maduramycin from Dr R. Dickey, United States Food and Drug Administration, Alabama, USA. Brevetoxin B and kabiramide C were kindly provided by Dr K. Nakanishi, Columbia University, NY, USA and Dr R. Andersen, University of British Columbia, respectively.

2.2. *Sources of protein preparations*

The biological activity of the marine toxins was assayed by the ability of these compounds to inhibit dephosphorylation of ^{32}P-

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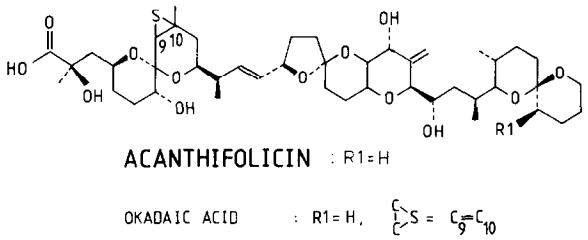

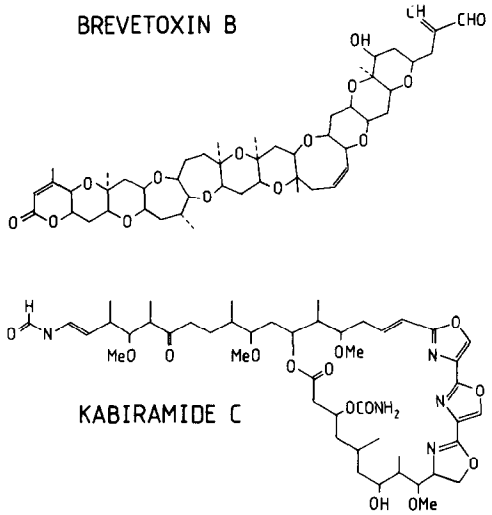

Fig. 1. Structures of acanthifolicin, brevetoxin B and kabiramide C. Acanthifolicin differs from okadaic acid in having an unusual episulphide functionality at the 9–10 carbon position, instead of a C=C bond. Dinophysistoxin-1 is methylated at the 35-carbon position.

radiolabelled phosphorylase *a* by protein phosphatases in the standard phosphorylase phosphatase assay [11]. Homogeneous PP1/2A catalytic subunits were purified from rabbit skeletal muscle [12] and generously provided by the United Kingdom Medical Research Council Protein Phosphorylation Group, Dundee (Professor P. Cohen). Radiolabelled phosphorylase *a* was prepared from phosphorylase *b* (Boehringer-Mannheim) by phosphorylation with ^{32}P-γ-ATP (Amersham) and phosphorylase kinase (Sigma). Prior to use, phosphorylase kinase and phosphorylase *b* preparations were routinely preincubated with 25 mM NaF to inhibit endogenous phosphatase activity.

3. RESULTS

3.1. *Effect of acanthifolicin, acanthifolicin methyl ester and methyl okadaate on protein phosphatase activity*

Acanthifolicin inhibited protein phosphatase-2A (PP2A) with slightly less potency than okadaic acid (Fig. 2) with an IC_{50} = 1.0 nM vs 0.2 nM, respectively. Full inhibition was achieved at 5.0 nM. Methyl esterification of the single carboxyl group on acanthifolicin decreased the potency of inhibition by

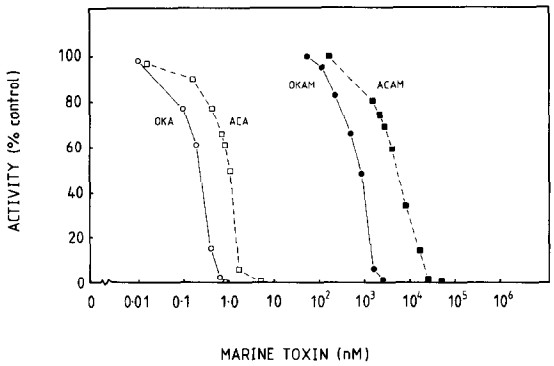

Fig. 2. Inhibition of protein phosphatase-2A catalytic subunit by acanthifolicin (□---□, ACA), acanthifolicin methyl ester (■---■, ACAM), okadaic acid (○—○, OKA) and methyl okadaate (●—●, OKAM).

5000-fold, acanthifolicin methyl ester inhibited PP2A with an IC_{50} = 5.0 μM, complete inactivation occurring at 50.0 μM. Similar results were achieved with methyl okadaate, which inhibited PP2A with an IC_{50} = 900 nM. Increasing the size and hydrophobicity of the group blocking the carboxyl function of okadaic acid had no further effect on activity since the 9-anthryldiazomethyl (ADAM) ester of okadaic acid was almost equally as ineffective as methyl okadaate as a PP2A inhiibtor (data not shown).

Acanthifolicin inhibited protein phosphatase-1 (PP1) with similar potency to okadaic acid (Fig. 3), the IC_{50} values being 20 and 19 nM, respectively. Full inhibition was achieved at 500 nM. Acanthifolicin methyl ester had very little effect on PP1 activity, even at concentrations as high as 250 μM.

3.2. *Effect of dinophysistoxin-1 and other polyether toxins on protein phosphatase activity*

The effect of dinophysistoxin-1 on protein phosphatase activity was determined since this compound is also a potent tumour promoter and is frequently found in the toxic profile of diarrhetic shellfish poisoning [13]. Dinophysistoxin-1 inhibited PP1 and PP2A with similar potency to okadaic acid, the IC_{50} values being 55 and 0.6 nM, respectively.

It was also of interest to examine the effects of other hydrophobic marine polyether and macrolide toxins on protein phosphatase activity. The C_{42} benthic marine poison brevetoxin B (Fig. 1), from the dinoflagellate *Ptychodiscus brevis*, resembles okadaic acid in size, hydrophobicity and structure, being composed of a contiguous polyether fused ring structure. The C_{36} macrolide kabiramide C is a strongly cytotoxic sponge metabolite from *Halichondria* sp. and was of particular interest since it reportedly inhibits phorbol ester induced inflammatory responses (R. Andersen, personal communication).

Brevetoxin B and kabiramide C had no effect on PP1 activity even at concentrations as high as 350 μM.

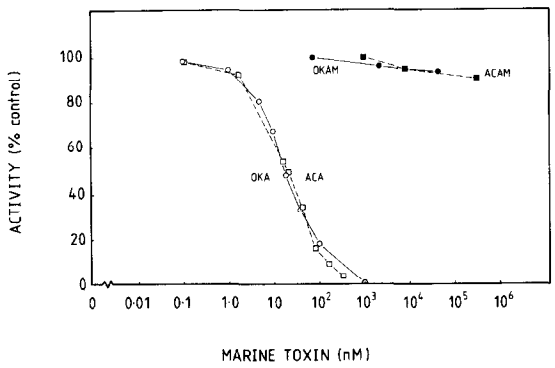

Fig. 3.

These compounds were slightly active against PP2A, causing 30 and 20% inhibition, respectively, at 350 μM in the phosphorylase *a* phosphatase assay.

4. DISCUSSION

Dinophysistoxin-1 and okadaic acid are powerful tumour promoters of comparable potency to phorbol esters [5]. Inhibition of PP1/PP2A probably underlies acceleration of tumour formation (reviewed in [2]) and not surprisingly, the potency of dinophysistoxin-1 as a phosphatase inhibitor is consistent with this notion. Similarly, acanthifolicin will likely be as powerful a tumour promoter as okadaic acid/dinophysistoxin-1 and by analogy could prove to be a diarrhetic shellfish poison [13].

Methyl esterification of the single carboxyl group on acanthifolicin (and okadaic acid) drastically reduced the potency of these compounds as protein phosphatase inhibitors, demonstrating that the region comprising this carboxyl group is important for effective inhibition of PP1/2A. This finding may facilitate a starting point for rational design of smaller synthetic protein phosphatase inhibitors. It has been reported that okadaic acid tetramethylether (methylated at 2-, 7-, 24- and 27-carbon hydroxyl groups) does not promote intracellular hyperphosphorylation in human fibroblasts and is not active as a tumour promoter [14,15]. It is presently not known how these methylations contribute to loss of biological activity.

Pure acanthifolicin/okadaic acid methyl esters may prove to be effective as specific inhibitors of PP2A at 1–10 μM concentrations in vitro and perhaps in vivo. In the latter instance, the fluorescent ADAM okadaic acid ester may be particularly useful (provided it is not rapidly hydrolysed by esterases) since it may facilitate in vivo intracellular localization studies.

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