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Tautomycin from the bacterium *Streptomyces verticillatus*

Another potent and specific inhibitor of protein phosphatases 1 and 2A

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Tautomycin inhibited the catalytic subunits of protein phosphatase-1 ($K_{i_{app}} = 0.16$ nM) more potently than protein phosphatase 2A ($K_{i_{app}} = 0.4$ nM), and the native forms of these enzymes in mammalian, protozoan and plant extracts were inhibited in a similar manner. Protein phosphatase 2B was inhibited 10000-fold less potently, while two other phosphatases and six protein kinases were unaffected at 10 μM. Okadaic acid prevented the binding of tautomycin to protein phosphatase 2A, indicating a common binding site for both inhibitors. The different relative potencies of tautomycin and okadaic acid for protein phosphatases 1 and 2A suggest that parallel use of both inhibitors may help to identify physiological substrates for each enzyme.

Tautomycin; Okadaic acid; Microcystin; Protein phosphatase; Tumour promoter

1. INTRODUCTION

Over the past few years two potent toxins produced by aquatic microorganisms have been shown to exert their effects by inhibiting protein phosphatases 1 and 2A (PP1, PP2A), two of the major protein serine/threonine phosphatases of eukaryotic cells. The first is okadaic acid [1], a fatty acyl polyketide produced by marine dinoflagelates which accumulates by filter feeding in the digestive glands of molluscs and marine sponges, such as *Halichondria okadaii* from which it was first extracted. It is a potent tumour promoter and one of the causative agents of diarrhetic seafood poisoning (reviewed in [2]). The second is microcystin-LR, a cyclic heptapeptide produced by certain species of the cyanobacteria *Microcystis, Oscillatoria* and *Anabaena*, which is a potent hepatotoxin [3,4]. Although both compounds have quite different chemical compositions, their binding to PP1 and PP2A is mutually exclusive, suggesting that they may interact at the same site on each enzyme [3]. Similarly, interaction of PP1 with the small thermostable proteins that inhibit this enzyme specifically, inhibitors 1 and 2, prevents the subsequent binding of microcystin-LR [3]. Thus all known substances that are potent inhibitors of PP1 and/or PP2A may exert their effects by binding to the same sites on these enzymes. The sensitivity of PP1 and PP2A to okadaic acid and microcystin-LR has been remarkably conserved and is virtually identical in eukaryotes as diverse as mammals, yeast and higher plants [3,5,6].

The soil bacteria *Streptomyces* make a vast array of structurally diverse compounds, some of which bear a resemblance to okadaic acid and microcystin. Many are polyketides (e.g. [7]) while others are peptides which, like microcystin, may contain both D- and L-amino acids and linkages other than α,α-peptide bonds (reviewed in [8]). Recently, a polyketide called tautomycin, produced by *Streptomyces griseochromogenes* and *Streptomyces verticillatus*, was purified and characterised [9]. When drawn following its structural formula, the backbone of tautomycin matches that of okadaic acid in the region from carbon-1 to carbon-16 (Fig. 1). Moreover, some reported effects of tautomycin on cells are similar to those produced by okadaic acid. For example, tautomycin is toxic to all eukaryotic cells tested [10] and rapidly induces bleb formation on the surface of human chronic leukemia cells, similar to those induced by phorbol dibutyrate [11]. Tautomycin, like okadaic acid, does not compete with tumour-promoting phorbol esters for binding to cells, and does not activate protein kinase C significantly in vitro [11]. Taken together these observations suggested that tautomycin, like okadaic acid, might be a protein phosphatase inhibitor. In this paper we show that tautomycin is indeed a potent inhibitor of PP1 and PP2A and that tautomycin and okadaic acid compete for binding to PP2A. However, unlike okadaic acid and microcystin, tautomycin has a higher affinity for PP1 than PP2A.

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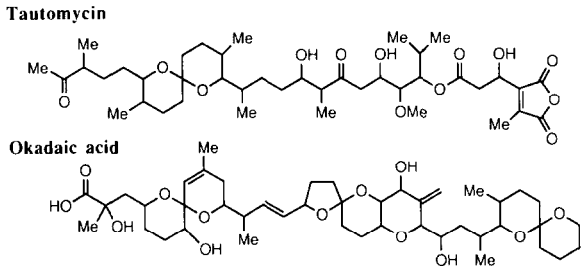

Fig. 1. Structural formulae of tautomycin and okadaic acid.

2. MATERIALS AND METHODS

Tautomycin, a kind gift from Dr K. Isono (Antibiotics Laboratory, Institute of Physical and Chemical Research, Saitama, Japan), was dissolved in dimethyl sulphoxide to give a 5 mM solution and further diluted in aqueous buffers before use. Okadaic acid was generously provided by Dr Y. Tsukitani (Fujisawa Pharmaceutical Co., Japan). Other materials and methods were identical to those described in [3].

3. RESULTS AND DISCUSSION

The dephosphorylation of glycogen phosphorylase (10 μM) by the purified catalytic subunits of PP1 and PP2A from rabbit skeletal muscle was potently inhibited by tautomycin. In the standard assay PP1 and PP2A were completely inhibited at 3 nM and 30 nM, respectively. PP2B was inhibited over 10000-fold less strongly, while PP2C was not inhibited at all (Fig. 2). Since the concentrations of tautomycin required for inhibition were similar to those of PP1 and PP2A in the assays, IC_{50} values were determined over a range of phosphatase concentrations. These experiments gave apparent K_i values of 0.16 nM and 0.4 nM for inhibition of PP1 and PP2A, respectively, after extrapolation to infinite dilution of enzyme (Fig. 3).

The specificity of tautomycin was examined further by testing its ability to inhibit other protein phosphatases and protein kinases. The tautomycin (10 μM) was without effect on calf intestinal and potato acid phosphatases, cyclic AMP-dependent protein kinase, protein kinase C, the AMP-activated protein kinase, casein kinase-1, casein kinase-2 and glycogen synthase kinase-3.

The specificity of tautomycin for PP1 and PP2A, but not PP2B or PP2C, was similar to okadaic acid and microcystin-LR and apparent K_i values extrapolated to infinite dilution are compared in Table I. Although all three inhibitors were effective in the nanomolar or subnanomolar range, tautomycin inhibited PP1 more strongly than PP2A, whereas the reverse was true for okadaic acid and microcystin.

We have shown previously that the binding of okadaic acid to PP2A prevents the subsequent binding of microcystin to PP2A [3] and the same experiments were employed to demonstrate that the binding of tautomycin and okadaic acid to PP2A are also mutually exclusive. Thus, the inhibition of PP1 by tautomycin was examined in the presence of PP2A that had first been inactivated by incubation with NaF and PP_i (see legend to Fig. 4). This caused an increase in the IC_{50} for tautomycin inhibition of PP1, implying that PP2A, although inactive, could nevertheless compete with PP1 for tautomycin under these assay conditions. However, the increase in IC_{50} was abolished by preincubating inactive PP2A with okadaic acid (Fig. 4), using a concentration in the assay (1.5 nM) sufficient to form a complex with PP2A, but too low to cause significant inactivation of PP1 [12]. These experiments demonstrated that the binding of okadaic acid to PP2A prevented the subsequent binding of tautomycin.

The catalytic subunits of PP1 and PP2A are complexed to regulatory and/or targetting subunits in vivo [13]. The active forms of PP1 and PP2A present in dilute extracts of rat liver or *Brassica napus* (rape) seeds were also strongly inhibited by tautomycin (data not shown). However, when diluted to the same activities (0.06–0.08 mU/ml in the assays), the IC_{50} values were

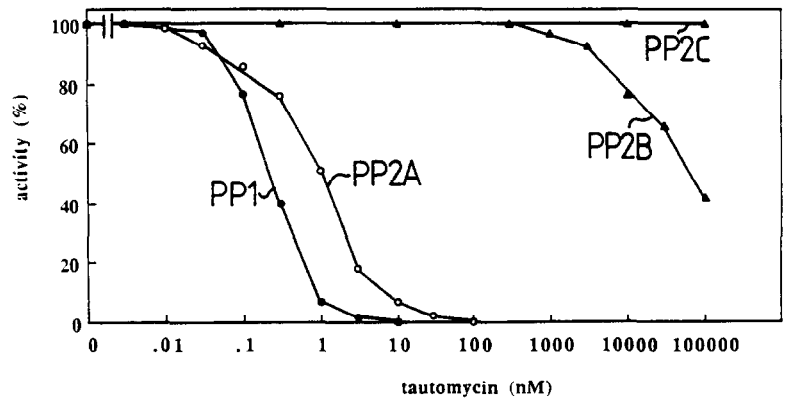

Fig. 2. Inhibition of different protein phosphatases by tautomycin. The catalytic subunits of PP1 (●) and PP2A (○) from rabbit skeletal muscle were both assayed at 0.24 mU/ml, respectively, using ^{32}P-labelled glycogen phosphorylase as substrate, PP2B from bovine brain (▲) at 0.05 mU/ml using ^{32}P-labelled phosphorylase kinase and PP2C from rabbit skeletal muscle (△) at 0.035 mU/ml using ^{32}P-labelled casein. For details of the assays and definition of enzyme units see [3].

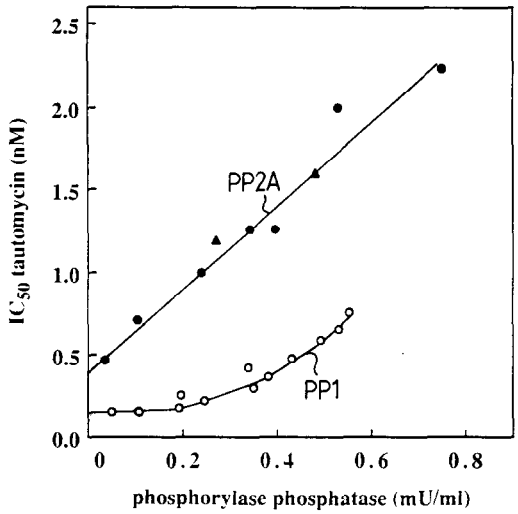

Fig. 3. Effect of protein phosphatase concentrations on inhibition by tautomycin. PP1 (○) and two different preparations of PP2A (●,▲).

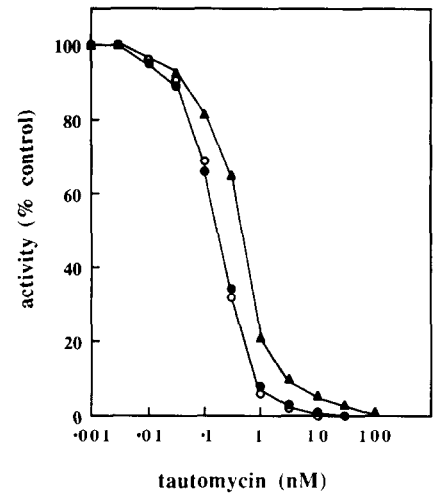

Fig. 4. Okadaic acid prevents the interaction of tautomycin with PP2A. The purified catalytic subunit of rabbit skeletal muscle PP2A (0.6 ml, 575 mU/ml) was inactivated by incubation for 16 h on ice in 50 mM Tris-HCl, pH 7.0 (4°C), 0.1 mM EGTA, 0.1% 2-mercaptoethanol (Solution A) containing 100 mM NaF, 5 mM sodium pyrophosphate and 1 mg/ml bovine serum albumin, and then passed through a 17×1.2 cm column of Sephadex G50 Superfine equilibrated in Solution A to remove fluoride and pyrophosphate. The inhibition of the catalytic subunit of PP1 (0.056 mU/ml) by tautomycin was then examined using ^{32}P-labelled glycogen phosphorylase as substrate, in the presence (▲) and absence (●) of the G50 eluate (diluted a further six-fold in the assay). The open circles show the effect of preincubating the G50 eluate with 9 nM okadaic acid (diluted to 1.5 nM in the assay, a concentration that causes less than 5% inhibition of PP1). Similar results were obtained in two separate experiments.

2-4-fold higher than those observed with the free catalytic subunits (Fig. 3). This is probably explained by the presence of regulatory/targeting subunits which suppress catalytic activity [13]. Thus the molar concentrations of the native forms of PP1 and PP2A in the assays will be higher than the catalytic subunits and therefore require higher concentrations of tautomycin for inhibition. Similar observations have been made for microcystin-LR [3].

Tautomycin inhibited PP1 purified from the ciliary membranes of the protozoan *Paramecium tetraurelia* [14] with an IC_{50} value of 2 nM. However, like microcystin and okadaic acid [3,14], tautomycin failed to inhibit the PP2A-like enzymes of *Paramecium* and did not inhibit *Paramecium* PP2C.

After these studies had been completed, we learned that Isono and coworkers have found that tautomycin competes with okadaic acid for binding to a particulate fraction prepared from mouse brain. They have also shown that tautomycin inhibits (IC_{50} = 2.5 nM) the dephosphorylation of histone by a partially purified protein phosphatase preparation from mouse brain (presumably PP1, PP2A, or a mixture of these enzymes) and that tautomycin (40 μM) stimulates protein phosphorylation when added to intact human leukaemia K562 cells in a similar manner to 40 μM okadaic acid (personal communication). The observation that tautomycin can enter cells suggests that, like okadaic acid ([15], reviewed in [2]) it may be useful in identifying physiological substrates for PP1 and PP2A and for identifying cellular processes that are controlled by phosphorylation. However, as the relative potency of tautomycin for PP1 and PP2A differs markedly from that of okadaic acid (Table I), the parallel use of both compounds over a wide concentration range may provide clues as to which substrates are dephosphorylated by PP1 and which are dephosphorylated by PP2A in vivo.

Table I

Apparent K_i values for inhibition of protein phosphatases 1 and 2A by tautomycin, okadaic acid and microcystin-LR

	Apparent K_i (nM)	
	PP1	PP2A
Tautomycin	0.16	0.4
Okadaic acid	10	~0.03
Microcystin-LR	0.06	<0.01

Apparent K_i values were calculated by extrapolating plots of IC_{50} against mU/ml to zero phosphatase concentration. Apparent K_i values were calculated from the data in Fig. 3 (tautomycin) [12] (okadaic acid) and [3] (microcystin-LR). Assays were performed at a single substrate concentration (10 μM glycogen phosphorylase)

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