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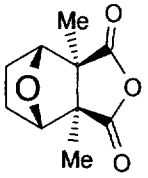
7-OXA[2.2.1]BICYCLOHEPTANE-2,3-DICARBOXYLIC ACID DERIVATIVES AS PHOSPHATASE INHIBITORS[1]

Albert Enz, Gerhard Zenke and Esteban Pombo-Villar *

Novartis Pharma Ltd, CH-4002 Basel, Switzerland

Abstract: Cantharidin (**1**) as well as norcantharidin or endothall (**2**) have been described to be inhibitors of protein phosphatase 2A, but were reported to be inactive as inhibitors of calcineurin. Using an HPLC-assay, we have discovered that **1** and **2** are inhibitors of calcineurin-dependent (appKi = 10.8 and 3.26 μM, respectively) dephosphorylation of a phosphopeptide derived from the RII subunit of c-AMP dependent kinase, H-DLDVPIPGRFDRRVS(PO_4)VAAE-OH (**7**), which is commonly used in calcineurin assays. The phosphatase inhibitor **6** also inhibits the production of IL-2 in Jurkat cells, a calcineurin dependent process, with IC_{50} = 4.7 μM, indicating that some of the biological activity of this substance class may be due to calcineurin inhibition, in addition to the established PP2A inhibition. © 1997 Elsevier Science Ltd.

Calcineurin (PP2B) is a calcium and calmodulin regulated protein phosphatase which is involved in a large variety of biological processes.[2] In particular, the discovery that PP2B is the biological target of both cyclosporin A (CsA) and FK-506 in immunosuppression has enhanced the interest in finding novel PP2B inhibitors. Both CsA and FK-506 must, however, form complexes with binding proteins (immunophillins) to exert their inhibitory action on PP2B. Casida and his group identified a cantharidin-binding protein from mouse liver as protein phosphatase 2A (PP2A), and showed that both cantharidin (**1**) and the herbicide endothall (**2**) inhibited this enzyme, using phosphorylase kinase as a substrate.[3]



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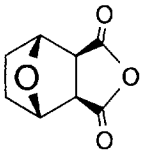

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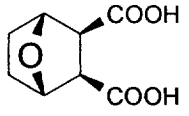

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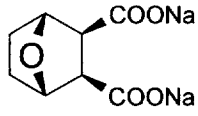

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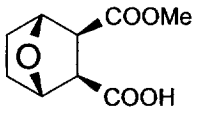

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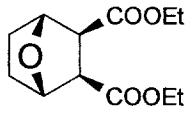

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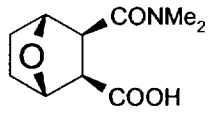

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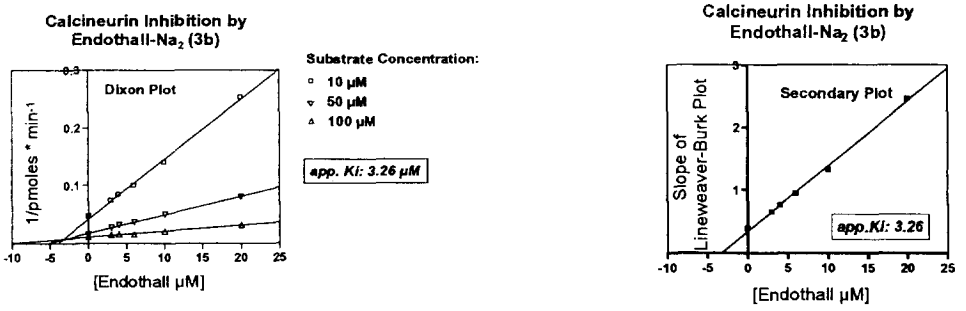
However, they also reported that neither **1** nor **2** inhibited the PP2B-dependent dephosphorylation of the phosphopeptide **7**, H-DLDVPIPGRFDRRVS(PO_4)VAAE-OH,[3] in concentrations up to 30 or 60 μM, respectively.[4] Honkanen[5] also observed the effect of **1** as inhibitor of PP1 and PP2A dephosphorylation of

* E-Mail: esteban.pombo@pharma.novartis.com; Telefax: +41-61-3249794

phosphohistone, but only observed a marginal effect on PP2B dephosphorylation of p-nitrophenyl phosphate (pNPP) at high concentrations. In view of the large sequence identity of the catalytic subunits of PP2A and PP2B, we decided to reinvestigate these puzzling results, and study the interaction of these and analogous compounds with PP2A and calcineurin. Even if both enzymes may be assayed using pNPP as a substrate,[6] we considered that a more relevant comparison of inhibitors could be obtained if they were assayed using the same phosphopeptide **7** as substrate.

Compounds **1-5** are either commercially available or readily synthesized by literature procedures.[7] Although **6** is thermally unstable,[8] it can be readily obtained.[9] For the enzyme assays, stock solutions of the compounds were prepared in DMSO (10^{-2} M) and further diluted with water to the appropriate concentrations. The final concentration of DMSO was 0.02%. For the cellular assays, stock solutions (5 x 10^{-2} M) in DMSO were stored at -20°C.

Figure 1 Dixon Plot and Secondary Plot of **3b.**



Calcineurin Inhibition

Calcineurin was purchased from UBI (Lake Placid, U.S.A.). Calcineurin phosphatase activity was determined by the HPLC assay previously described[10] by monitoring the dephosphorylation of the 19-amino acid phosphopeptide **7**, H-DLDVPIPGRFDRRVS(PO_4)VAAE-OH. The non-radioactive HPLC assay developed by us allows the simultaneous observation of the substrate phosphopeptide and the product dephosphorylated peptide. We have used this assay to characterise the PP2B inhibition of the CsA/cyclophilinA complex, microcystin-LR, as well as of okadaic acid.[11] The calcineurin preparation showed Km=25 µM using phosphopeptide **7** as a substrate. Okadaic acid inhibited the dephosphorylation of **7** with an apparent Ki of 2.4 µM, and either **2** or **3**, showed appKi = 3.26 µM, as can be deduced from the Dixon plot and the secondary plot obtained from the Lineweaver-Burk plot (Figure 1). The inhibitory properties of compounds **1-6** were analysed in similar fashion, and the results are summarised in Table 1. From these enzyme kinetics experiments, the inhibition is seen to be of linear-mixed-type

(comprising both competitive and non-competitive properties). An explanation for this behaviour may be that the inhibitors interact with both the free enzyme and with the enzyme-substrate complex. Care, however, must be exercised in the interpretation of these kinetic results in molecular terms, until further studies are performed. [12]

Inhibition of PP2A

PP2A catalytic subunit was purchased from UBI (Lake Placid, U.S.A.) and used without further purification. Enzyme activity was assayed under analogous conditions to those described by Takai and Mieskes[13], but by using the same substrate as in the PP2B assay described above.[14] The Km of PP2A with substrate **7** was 130 μM. The type of inhibition observed was linear-mixed, similar to that observed with PP2B. The results of PP2A activity are shown in Table 1.

Table 1 PP2B and PP2A inhibition

compound	PP2B Ki μM	PP2A Ki μM
1	10.8	0.065
2	3.33	0.2
3b	3.02	0.17
4	>100	n.d.
5	>100	n.d.
6	2.78	0.18

A recent publication reports differences between the inhibition of the dephosphorylation of phosphorylase a by heterotrimeric PP2A by **2** and **3a**.[15] We have not observed any difference in the inhibitory behaviour of the anhydride **2** and the disodium salt **3b**. It is perhaps not surprising, given that the anhydrides are sparingly soluble in the assay buffer, and presumably the dissolved species is the diacid. The monoester **4** and diester **5** are not active as inhibitors of PP2B, but the monoamide **6** is again similar in potency to **2** and **3**.

Endothall **2** has been established *in vitro* as an inhibitor of PP1 and PP2A (PP1 IC_{50} = 5000 nM, PP2A IC_{50} = 970nM),[3] and supporting evidence has been obtained to indicate that this may be important to elucidate its action *in vivo*.[16] From our results it is also clearly an inhibitor of PP2B, albeit at higher concentrations. Use of the same phosphopeptide substrate for both PP2A and PP2B shows that endothall is a potent inhibitor of both phosphatases. In fact, some of the effects of this substance class in biological systems may be due to PP2B inhibition.

The transcription of IL-2 in lymphocytes has been shown to be a PP2B-dependent process.[17] The effects of compounds **1-6** on the transcription and release of IL-2 were tested using a reporter-gene assay (RGA) where β-galactosidase stably transfected into Jurkat cells is placed under the control of the IL-2 promoter, and the activity of the β-galactosidase is monitored by the fluorescence generated by the cleavage of 4-methylumbelliferyl-β-D-galactoside as described previously.[18] The constitutively active thymidine kinase promoter driving the expression of

the same reporter gene is used as control for cytotoxicity. Additionally, as a measure for the endogenous IL-2 gene activity, secretion of IL-2 was monitored in supernatant, by means of a proliferation assay using the IL-2-dependent mouse T cell line (CTLL) as previously described.[19] The results for **2**, **3b**, **6** and cyclosporin A are shown in **Table 2**. Compound **6**, showed inhibition of both IL-2 transcription and release at concentrations comparable to the order of magnitude of its Ki for PP2B. The higher activity of **6** compared to **2** and **3b** in this assay may be due to improved penetration into the cytosol. Interestingly, **6** also shows a 20-fold selectivity window between its inhibition of IL-2 transcription and release, and its cellular toxicity. Although we cannot rule out that **6** may gradually hydrolize to diacid **3** under the phosphatase assay conditions, the compounds show different activity in the Jurkat cell assay.

Table 2. Effects on IL-2 production in Jurkat cells[a]

Compound	β–galactosidase IL2 RGA IC_{50} μM	Inhibition of IL2 secretion IC_{50} μM	β–galactosidase thymidine kinase RGA- IC_{50} μM
2	>22 , 100	n.d.	100
3b	>100, >100	n.d.	42
6	4.7 +- 1.2[b]	5	100
cyclosporin A (CsA)	0.008 +- 0.003[b]	0.007	>1

[a]) IC_{50} values of individual experiments. [b]) Mean and standard deviation of 3 experiments

A preliminary study indicated possible immunosuppressive activity of **2**, indicating inhibition of lymphocyte proliferation and dose-dependent inhibition of a mixed-lymphocyte reaction.[20] Both of these processes are strongly affected by inhibition of PP2B,[21] and even if other mechanisms involving unspecific toxicity may also be involved, such inhibition is consistent with the effects we describe in Jurkat cells. An *in vivo* study of **1**, however, reports an increase in the spleen lymphocyte proliferation and of IL-2 and IL-1 production in mice, after 0.75 and 1.5 mg/kg applied i.p. three times every other day.[22] This, however, may be a secondary reaction after tissue damage, as the irritant and inflammatory properties of **1** have been well documented.[23] Clearly, further studies are needed to determine the relative importance of PP1, PP2A and PP2B inhibition in the biological effects of this class of compounds.

Inhibition of PP2A is associated with toxicity to most organisms, but this property may be useful in cancer chemotherapy.[24,25] Inhibition of PP2B, is associated with immunosuppression, and may result in a larger variety of therapeutic applications. The compounds used in this study are substantially weaker inhibitors PP2B than the cyclosporin/cyclophilin complex, but may be acting by direct interaction with the active catalytic site of the enzyme. Recent publications detailing the crystal structures of the several related protein phosphatases[26] provide new possibilities by using this readily accessible substance class for the discovery of small molecules which may be

selective inhibitors of the protein phosphatases. In the course of our work, a publication has appeared showing the use of the PP2B crystal structure in the design of endothall analogues with increased PP2B inhibitory activity. [27]

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9. A 10% solution of dimethylamine in THF (13 ml, 29.7 mmol) was added dropwise to a cooled (5°C) solution of **2** (5.0 gm, 29.7 mmol) in CH_2Cl_2 (15 ml), and the resulting solution was left to stand at RT for 48 h. The clear colourless solution was evaporated and the solid residue was recrystallized (acetone-CH_2Cl_2-pentane). The colourless crystals were dried (0.5 mBar at 40°C, 1h): (5.3 g, 83.7%), m.p.128-129 °C. Expected for $C_{10}H_{15}NO_4$: C 56.33, H 7.09, N 6.57, O 30.01. Found: C 55.98, H 6.95, N 6.48, O 30.41.MS (CI, CH_4): 242 (5, $[M+C_2H_5]^+$)214 (50, $[M+H]^+$), 196 (100) ^{1}HNMR (d6DMSO): δ 1.5 (m, 4H), 2.74 (s, 3H), 3.94 (s, 3H), 3.98(d, J = 11 Hz, 1H), 3.17 (d, J = 11 Hz, 1H), 4.60 (br s, 1H), 4.67 (d, J = 5 Hz, 1H); IR (KBr):1732, 1615, 1170

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