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THE ROLE OF ARGININE IN INTERACTIONS OF MICROCYSTINS WITH PROTEIN PHOSPHATASES 1 AND 2A

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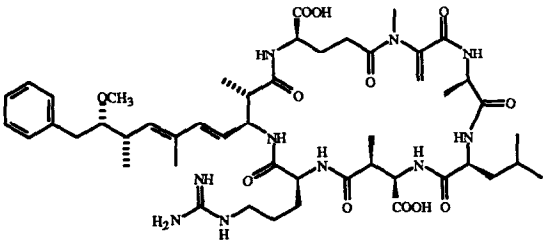
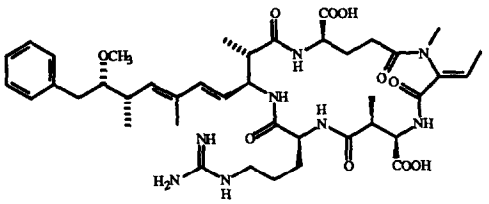
Abstract: Comparison of the enzyme inhibition and receptor binding activity of microcystin-LR and microcystin-LA has shown that the arginine residue in microcystin-LR does not significantly contribute to biological activity. This data allows a refinement of a receptor binding model of the okadaic acid class of protein phosphatase inhibitors.

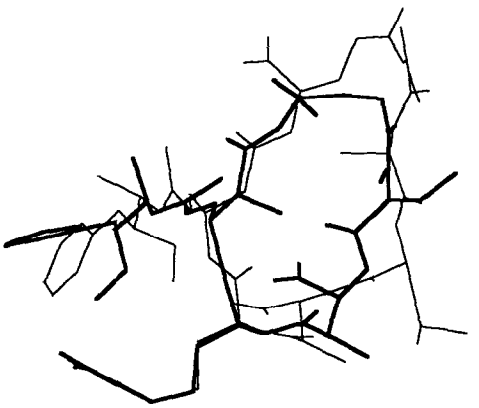
We have recently reported that the computer generated minimized 3-dimensional structures of microcystin-LR (**1**) and nodularin (**2**), two cyclic peptides of the okadaic acid class of protein phosphatase inhibitors, have the same orientation of (2S,3S,8S,9S) 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Adda) with respect to both peptide rings.[1] These results are consistent with these two compounds inhibiting protein phosphatase 1 (PP1) and 2A (PP2A) activities with almost the same specific activity. The superimposition **3** shows a proximity of the Adda side chains, the Glu and D-*erythro*-β-methylisoAsp carboxylate groups and the peptide rings.[2] This microcystin-LR/nodularin model also shows that



1 ED_{50} (PP2A from mouse skin) 0.8nM[3]



2 ED_{50} (PP2A from mouse skin) 0.9nM[3]



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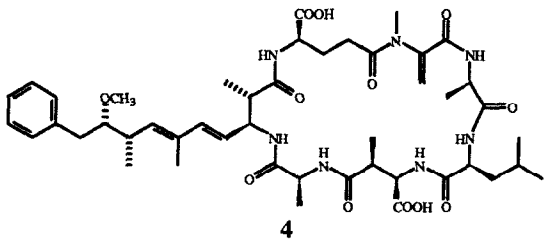
the Arg residues in microcystin-LR and nodularin occupy a common area. We now report experiments that determine the role of the Arg residue in inhibiting enzyme activity and inhibiting specific [^{3}H]okadaic acid binding to the receptors.

Microcystin-LR (**1**) is a member of the microcystin class of compounds which have the general structure cyclo(D-Ala-L-X-D-*erythro*-β-methylisoAsp-L-Y-Adda-D-isoGlu-N-methyldehydroAla) where X and Y are variable L-amino acids. The naturally occurring microcystin-LA (**4**) is identical to microcystin-LR except that the variable amino acid Y is alanine instead of arginine.[4] These two isomers provide a probe to determine the relative importance of the Arg residue to the biochemical and biological activities. **1** and **4** were found to inhibit the dephosphorylation of [^{32}P]phosphorylase a by protein phosphatase 1 with ED_{50}'s of 0.44 nM and 0.90 nM, respectively and protein phosphatase 2A with ED_{50}'s of 0.32 nM and 0.38 nM, respectively (Figure 1).[5]



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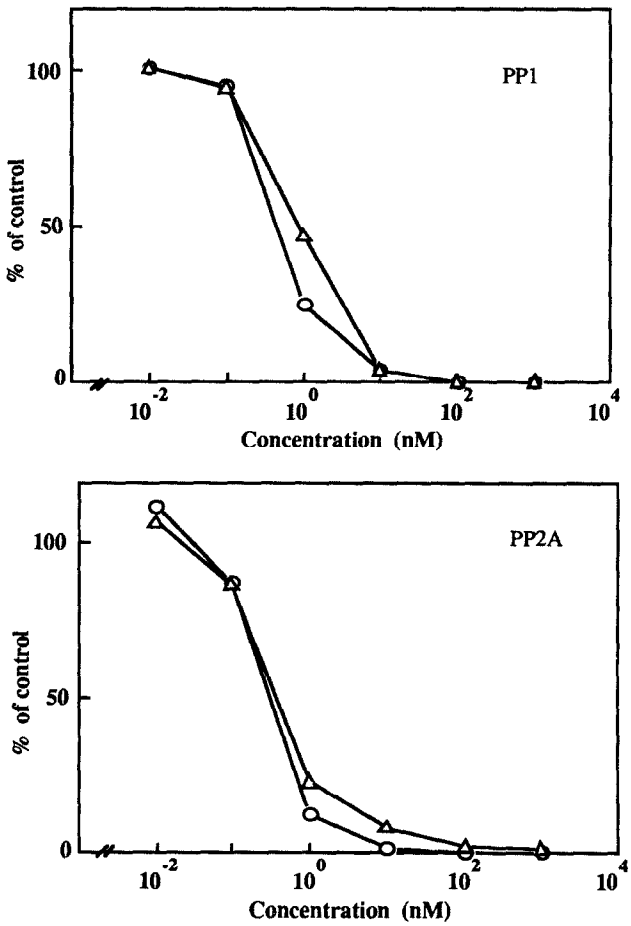

Figure 1. Inhibition of protein phosphatase activity
Effects of microcystin-LR (**1**, o) and microcystin-LA (**4**, Δ) on the activity of PP1 and PP2A partially purified from mouse brain. Assays were conducted using [^{32}P]phosphorylase a as substrate. Incubations were conducted for 10 min at 30 °C. Protein phosphatase activity was determined by the liberation of [^{32}P]phosphate into the supernatant after precipitation of protein with trichloroacetic acid. Each point represents the mean of duplicate measurements.

Microcystin-LR belongs to the okadaic acid class tumour promoters and induces tumour promotion in rat liver initiated with diethylnitrosamine.[6] The displacement of specific [^{3}H]okadaic acid binding to both protein phosphatase 1 and protein phosphatase 2A was determined. **1** and **4** were found to inhibit specific [^{3}H]okadaic acid binding to protein phosphatase 1 with ED_{50}'s of 7.8 nM and 10.6 nM, respectively and protein phosphatase 2A with ED_{50}'s of 5.2 nM and 10.0 nM, respectively (Figure 2).[7]

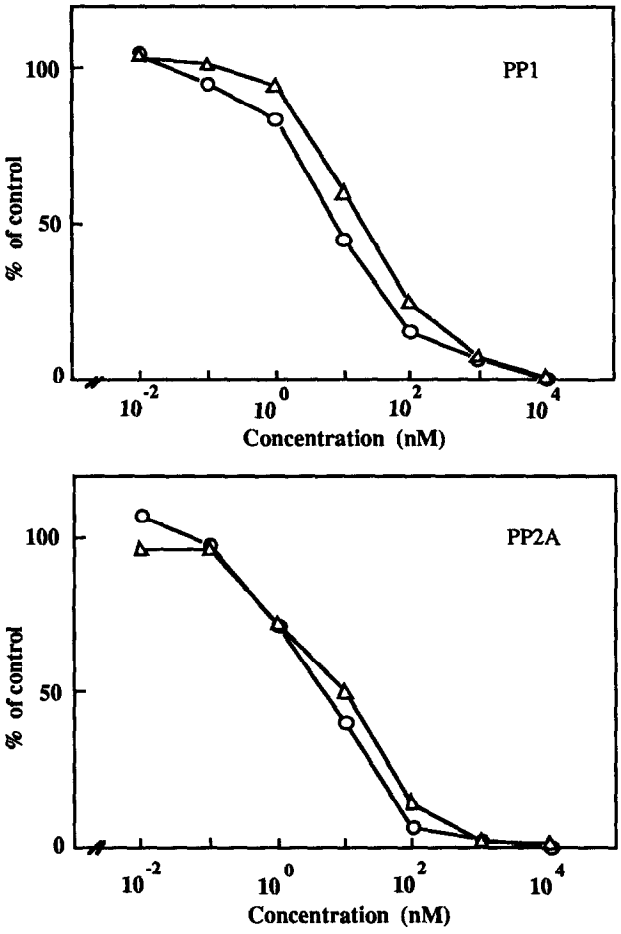

Figure 2. Inhibition of specific [^{3}H]okadaic acid binding.
Effects of microcystin-LR (**1**, o) and microcystin-LA (**4**, Δ) on the binding of [^{3}H]okadaic acid to PP1 and PP2A partially purified from mouse brain. Incubations were conducted for 2 h at 4 °C with 3 nM [^{3}H]okadaic acid. Non-specific binding was determined in the presence of 1.5 μM okadaic acid. Each point represents the mean of duplicate measurements.

The similar specific activity of microcystin-LR and microcystin-LA determines that the arginine residue does not significantly interact with the enzymes and demonstrates that the orientation with respect to the peptide ring of the Adda and the two acidic groups is most likely to determine enzyme / inhibitor interaction.

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References and Notes

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5. Protein phosphatases 1 and 2A were partially purified and assayed in a modification of the method previously reported in Nishiwaki, S.; Fujiki, H.; Suganuma, M.; Nishiwaki-Matsushima, R.; Sugimura. T. *FEBS Lett.* **1991**, *279*, 115.
Protein phosphatase 1 and 2A were isolated from mouse brain by DEAE-cellulose column chromatography using 50 mM Tris-HCl buffer (pH 7.4). Partially purified PP1 was eluted with buffer containing 0.1 M NaCl and partially purified PP2A was eluted with buffer containing 0.2 M NaCl. Enzyme activity was measured in 50 mM Tris-HCl buffer (pH 7.5) containing 100 μM EDTA, 5 mM caffeine, 0.1% 2-mercaptoethanol, 0.6 mg/mL BSA and [^{32}P]phosphorylase a. Inhibition of protein phosphatase activity was determined by incubation of [^{32}P]phosphorylase a (5 μg), protein phosphatase 1 (18.5 μg) or 2A (11.8 μg) and various concentrations of microcystins-LR or -LA for 10 min at 30 °C in 100 μL volume in duplicate. The reaction was terminated by addition of 100 μL of ice cold 50% trichloroacetic acid. After centrifugation an aliquot (150 μL) of supernatant was counted in Amersham Aquasol scintillant. Data expressed as percent inhibition with respect to a control (absence of competing compound) incubation.
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7. [^{3}H]Okadaic acid binding to protein phosphatases 1 and 2A were assayed as previously reported in Yoshizawa, S.; Matsushima, R.; Watanabe, M. F.; Harada, K.-I.; Ichihara, A.; Carmichael, W. W.; Fujiki, H. *J. Cancer Res. Clin. Oncol.* **1990**, *116*, 609.
Protein phosphatase 1 (100 μg) or protein phosphatase 2A (20 μg) was incubated with 10 nM [^{3}H]okadaic acid and various concentrations of microcystin-LR or microcystin-LA in 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM 2-mercaptoethanol at 4 °C for 2 h. Reaction was terminated by addition of cold acetone (-78 °C, 2.5 mL) and filtered on Whatman GF/C glass-fiber filters, precipitate was washed with cold acetone (12.5 mL). Specific [^{3}H]okadaic acid binding was estimated by the difference between total binding and nonspecific binding measured in the presence of a 500-fold excess of unlabeled okadaic acid. Data expressed as percent inhibition with respect to a control (absence of competing compound) incubation.