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Importance of the C28–C38 Hydrophobic Domain of Okadaic Acid for Potent Inhibition of Protein Serine-Threonine Phosphatases 1 and 2A

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Abstract—Okadaic acid is a potent inhibitor of select serine/threonine protein phosphatases. The importance of the C28–C38 hydrophobic domain of okadaic acid for inhibition of PP1 and PP2A was investigated. The hydrophobic domain is required but not sufficient for potent inhibition, and it also contributes to differential inhibition between PP1 and PP2A.

Protein serine/threonine phosphatases are an important class of enzymes responsible for dephosphorylating phosphoserine and phosphothreonine residues of protein substrates. As key players in signal transduction pathways, they are involved in the regulation of many essential processes, including muscle contraction, neurotransmission, cell cycle progression, cell division, cell adhesion, protein synthesis, and transcription.[1] Two key families of phosphatases are protein phosphatase 1 and 2A (PP1 and PP2A), which share significant sequence homology. Both are inhibited by a number of natural products, including okadaic acid, the microcystins, calyculin A, and tautomycin.[2] These occupy the same binding site on the phosphatases,[3] and the crystal structure of PP1 co-crystallized with microcystin-LR demonstrates that this binding site involves the active site of the protein.[4]

Okadaic acid (OA, **1**; Fig. 1), a polyether natural product originally isolated from the marine sponges *Halichondria okadai* and *Halichondria melanodocia*,[5] is the archetypal inhibitor of PP1 and PP2A. OA adopts a circular conformation in the solid state[5] and in solution,[6] which allows hydrogen bonding between the C1 carboxylate and the C24 hydroxyl (Fig. 2). This conformation is enforced by hydrogen bonding between the C2 hydroxyl and C4 pyranyl oxygen, the *trans* alkene at C14–C15, the configurations of ketals at C8 and C19, and the chair conformations of the oxane rings. Extended from the C1–C24 pseudomacrolide of OA is a hydrophobic domain that terminates in a 1,7-dioxaspirobicyclo[5.5]undecane moiety. Previous structure–activity studies that have been reported for OA suggest the importance of the carboxylic acid and some of the hydroxyl moieties for potent inhibition of PP1/PP2A.[7] However, naturally-occurring 7-deoxy OA (**2**) is nearly equipotent compared to OA.[7b] It seems likely that the carboxylate of OA is an essential mimic of phosphoryl moieties of normal substrates, and the C2, C24, and C27 hydroxyls may be involved in intra- or intermolecular hydrogen bonding interactions. The crystal structure of PP1/microcystin-LR, in particular, has spurred modeling studies by our group[8] and others[9] to investigate the binding of inhibitors to the protein phosphatases. Distinct models of OA with PP1 share some common features: the hydrophobic domain of OA is docked in a hydrophobic surface groove proximal to the active site, and the carboxylate is located near the cationic bimetallic well. These models provide some insight into the possible binding mode of OA, but are inconclusive. It has been hypothesized that the hydrophobic domain is an important recognition factor crucial for binding to the phosphatases. However, the specific role of the hydrophobic C28–C38 domain has not been thoroughly examined. Holmes and co-workers[10] report that a W206A mutation in a hydrophobic groove in PP1 significantly reduces binding of OA. These results

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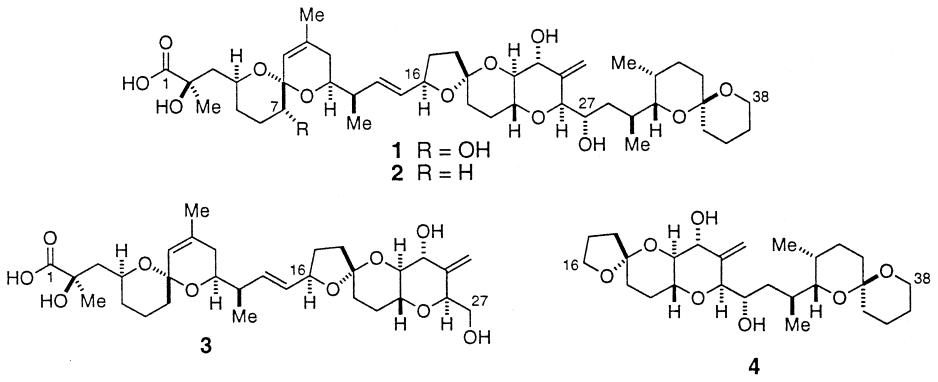

Figure 1. Structures of okadaic acid and analogues.

suggest that interactions between the hydrophobic domain of OA and the hydrophobic groove of PP1 may be important for binding. We are interested in further investigating the role of the OA hydrophobic domain by modifying the inhibitor instead of the proteins. Targeted truncated analogues of 7-deoxy okadaic acid are shown in Figure 1. The C1–C27 analogue **3** contains all of the functionality of the parent compound except for the hydrophobic domain, and was designed to investigate the necessity of this hydrophobic domain.[11] The C16–C38 analogue **4**[12] lacks the carboxylic acid of the parent compound, but maintains the hydrophobic domain, in order to investigate whether this portion is sufficient for inhibition. Herein are reported the results of an empirical study that demonstrates the importance of the C28–C38 hydrophobic domain of OA in the inhibition of PP1 and PP2A.

The syntheses of analogues of OA were based upon our convergent total syntheses of OA[13] and 7-deoxy OA.[14] 7-Deoxy OA analogues were targeted because of the similar inhibitory activity between OA and 7-deoxy OA. The synthesis of a protected C1–C27 portion of 7-deoxy OA (**3**) was previously reported.[15] Synthesis of compound **4** is shown in Figure 3. A cerium-mediated coupling of bromide **5**[13] and aldehyde **6**[13] gave a mixture of alcohols **7**. Stereochemical correction followed by debenzylation provided C16–C38 analogue **4**.[16]

With the series of truncated analogues in hand, it was necessary to examine the inhibitory activity of these compounds against the protein phosphatases. PP1 was obtained from a commercial source,[17] and PP2A was isolated from bovine myocardial tissue.[18]

The assay for inhibitory activity utilized a malachite green assay system, with a phosphopeptide substrate, KRpTIRR.[19] Results are summarized in Table 1. Against PP1, the IC_{50} for OA is 126 nM. With the omission of the hydrophobic domain, there is a significant decrease in activity, but the C1–C27 domain of 7-deoxy OA retains some activity, with an IC_{50} of 95 μM. This is approximately an 800-fold decrease in activity against PP1. The C16–C38 analogue **4** shows no detectable inhibitory activity against PP1, even at a

Table 1. Phosphatase inhibition by OA and analogues[a]

	IC_{50} (nM)	
Compd	PP1	PP2A
1	126	7
3	9.5×10^4	304
4	$>1\times10^6$	$>1\times10^6$

[a]Values given are averages of three trials. PP1 concentration was 10 nM, and PP2A concentration was 25 nM. Concentrations of OA and analogues ranged from 1 nM to 1 mM, depending on the compound.

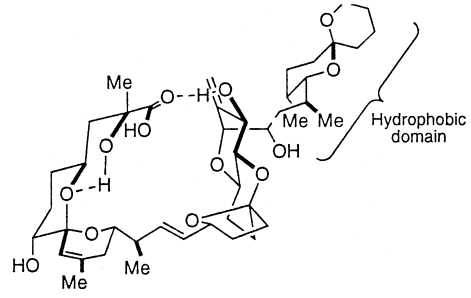

Figure 2. Circular conformation of OA.[6]

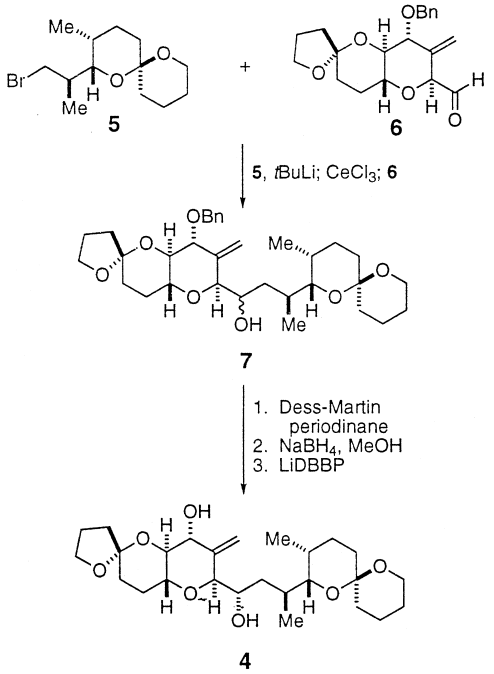

Figure 3. Synthesis of C16–C38 analogue **4**.

concentration of 1 mM. Against PP2A, the IC_{50} for OA in this system is 7 nM. In this case, the C1–C27 analogue retains significant inhibitory activity with an IC_{50} of 304 nM. Thus, omission of the C28–C38 hydrophobic domain results in an approximately 50-fold decrease in activity against PP2A. The other truncated analogue **4** again shows no detectable inhibitory activity against PP2A at a concentration of 1 mM. These results clearly demonstrate the importance of the C28–C38 hydrophobic domain for potent inhibition of the phosphatases. Moreover, it is shown that this domain is not sufficient for activity. Furthermore, a difference in specificity between PP1 and PP2A is highlighted, as omission of the hydrophobic domain has a more drastic effect on PP1 than PP2A. The hydrophobic domain is more crucial to the inhibition of PP1 than PP2A.

Modifications of a flexible total synthesis provided access to analogues of OA to investigate the importance of the C28–C38 hydrophobic domain for inhibition and binding of PP1 and PP2A enzymes. Enzymatic assays demonstrate that this domain is important, but not sufficient for potent inhibition. These results are consistent with modeling and mutagenesis studies, and are consistent with a model of OA binding in approximately its native circular conformation (Fig. 2) with the carboxylate binding at the cationic bimetallic active site and the hydrophobic terminus occupying a proximal hydrophobic groove. These studies indicate that occupancy of a hydrophobic surface groove proximal to the active site by the hydrophobic domain of OA contributes significantly to the overall binding affinity. In addition, binding of the hydrophobic domain appears to provide a factor for selective inhibition between the two phosphatases under study, as this moiety is more critical to inhibition of PP1 than PP2A. Further studies are underway to continue to elucidate the structural features of OA required for binding and potent, selective inhibition of PP1 and PP2A enzymes.

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