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STRUCTURE-BASED DESIGN OF NOVEL CALCINEURIN (PP2B) INHIBITORS

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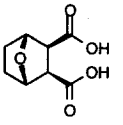
Abstract: The design, synthesis, and evaluation of small molecule, in vitro, inhibitors of human calcineurin is described. These ligands were derived from the known nonspecific phosphatase inhibitor endothall, and were modified to enhance binding and selectivity toward calcineurin using protein crystal structure information.

Calcineurin (protein phosphatase 2B [PP2B]) is a calcium and calmodulin regulated enzyme composed of a 59-kDa catalytic subunit (CnA) and a 19-kDa calcium binding subunit (CnB).[1] The catalytic subunit CnA shares extensive sequence homology with two other members of the serine/threonine protein phosphatase family, PP1 and PP2A.[2] Calcineurin has been identified as a key signaling enzyme in T-lymphocyte activation.[3] Inhibition of calcineurin in T-lymphocytes prevents the formation of active transcription factors, such as NF-AT and NF-IL2A, which are essential for interleukin-2 (IL2) gene expression.[4] Inhibition of calcineurin leads to the disruption of the cellular immune response, since IL2 is necessary for T-cell proliferation.[5]

The immunosuppressant drugs cyclosporin A and FK506 each bind to distinct intracellular receptors, referred to as the immunophilins.[6] The resulting drug-immunophilin complexes independently bind to and inhibit the protein phosphatase activity of calcineurin.[7] A great deal of effort has been devoted to the search for synthetic immunosuppressants which inhibit calcineurin through an immunophilin complex.[8] However, a more direct strategy has surfaced with the recent X-ray crystal structure determination of calcineurin.[9] We felt some advantages, in terms of toxicity and molecular simplicity, could be realized with catalytic site directed inhibitors of calcineurin. In the present communication, we wish to report the discovery of a series of molecules that bind tightly to calcineurin, as determined by inhibition of protein phosphatase activity.[10] These compounds also serve as a means to address ligand selectivity toward calcineurin over PP1 and PP2A.



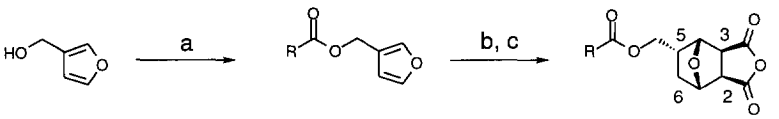
Endothall

The search for leads began with a survey of known direct inhibitors of protein phosphatases 1, 2A, or 2B.[11] For example, cantharidin,[12] endothall,[12(b),13] microcystins,[14] nodularin,[15] okadaic acid,[16] and tautomycin[17] were considered. We reasoned that the *exo,exo*-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid ring system of endothall would serve as an excellent starting point based on structural versatility and relative ease of synthesis compared with the other natural product inhibitors.[18] A model of an endothall derivative bound to the calcineurin active site was generated by computational docking experiments and with information extracted from a low resolution co-crystal structure.[19] Ligands were designed using this model and with clues obtained by studying interactions between the calcineurin autoinhibitory peptide and the catalytic domain.[9(b),20]

With this information in hand, we attempted to enhance ligand binding to calcineurin relative to endothall (Table 1). Moreover, using available PP1 crystal structure information and PP2A amino acid sequence homology data, we were prepared to address specificity toward calcineurin.[2, 21] According to our binding model, the dicarboxylic acid and the bridge head oxygen act as an anchor, interacting with the catalytic site metals and surrounding residues (Figure 1). For modification, our attention was drawn to the 5 and 6 position of the 7-oxabicyclo ring system (Scheme 1). 5-*endo* substitution appeared to provide directionality into a region of the protein with potential for reasonable binding interactions. Additionally, this substitution pattern allowed us to take advantage of some CnA specific residues in this area.

The substituted endothall derivatives were constructed by a Diels-Alder reaction between maleic anhydride and an appropriate 3-substituted furan. 3-Hydroxymethylfuran was coupled to various carboxylic acids to yield the necessary dienes for the cycloaddition. Hydrogenation of the resulting adduct gave the desired product, in high yield, as a mixture of optical isomers.[22] No attempt at resolution was made, although the binding model suggested only the enantiomer depicted below would bind effectively.

Scheme 1.



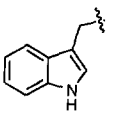
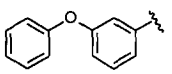
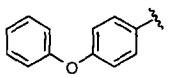
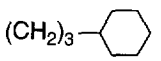
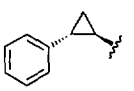
1a-j **2a-j**

Reagents and Conditions: (a) RCOOH, EDC·HCl, DMAP, CH_2Cl_2, 23 °C (b) Maleic anhydride, Et_2O, 23 °C (c) 10% Pd/C, H_2, DME.

Table 1. Inhibition of Calcineurin Phosphatase Activity by Synthetic Ligands.

Compound	R	$K_{i,\,app}$ (μM)
endothall	—	11.5
2a	Ph	11.0
2b	CH_2Ph	7.4
2c	$(CH_2)_2Ph$	3.7
2d	$(CH_2)_3Ph$	1.2
2e	$(CH_2)_4Ph$	2.3

Table 1 continued.

Compound	R	$K_{i,app}$ (μM)
2f		2.0
2g		1.8
2h		1.4
2i		1.0
2j		0.5

mixture of trans-cyclopropanes

Table 2. Comparison of Ligand Inhibitory Effect on PP1 and PP2B Activity.

Compound	PP2B $K_{i,app}$ (μM)	PP1 $K_{i,app}$ (μM)
endothall	11.5	4.0
2j	0.5	4.0

From the 5 position of the endothall core, we attempted to mimic an interaction observed between the bound autoinhibitory domain and the calcineurin active site. The interaction studied was between Phe-470 of the autoinhibitory peptide and a hydrophobic region of the active site specific to PP2B.[9(b)] We believe the terminal phenyl ring of ligand **2j** binds to this area of calcineurin with a similar orientation. This region, or phenylalanine pocket, is located adjacent to CnA Tyr-315 (Figure 1). According to our ligand binding model, the Tyr-315 hydroxyl forms a hydrogen bond with the ester functionality of the endothall-based ligands. In the case of PP1, the residue that corresponds to this tyrosine is phenylalanine, and in PP2A the residue is cysteine. The unconserved nature of this position could account for differences in the inhibitory effect of ligand **2j** on PP1 and PP2B, since no hydrogen bond opportunity is available with PP1 (Table 2). Although we do not have inhibition data for PP2A, it is known that endothall is a potent inhibitor of this enzyme.[12(b)] Perhaps the modifications made to the endothall core, along with the differences in binding regions, would result in diminished potency toward PP2A.

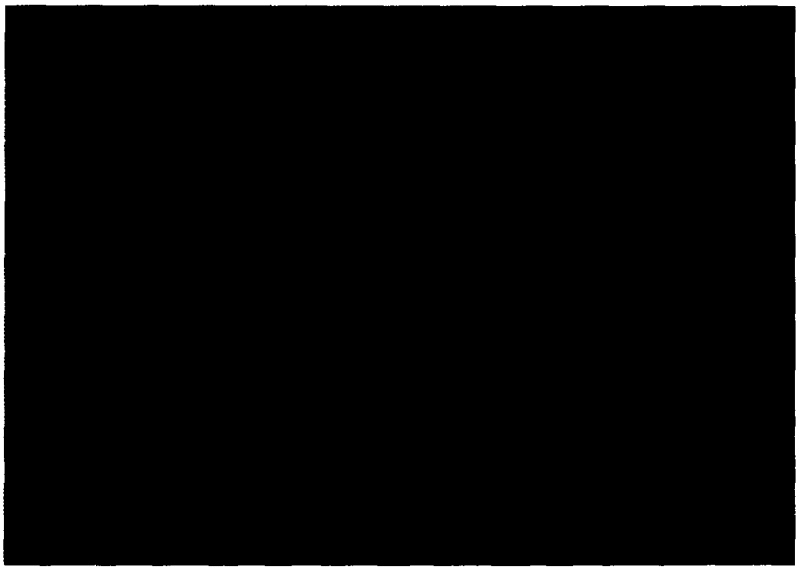


Figure 1. Binding model of ligand 2j (as the diacid, green) with the autoinhibitory domain phenylalanine ring overlaid (yellow). Catalytic site Zn^{2+} and Fe^{3+} displayed (brown spheres).

Incorporation of a trans-cyclopropylphenyl group clearly gave the best results (ligand **2j**, Table 1). Perhaps conformational preorganization, due to the rigid nature of this fragment, contributed favorably to binding. We also imparted significant PP2B selectivity with this substituent. Endothall was about three fold more selective for PP1 over PP2B, while endothall derivative **2j** had an eight fold preference for PP2B over PP1 (Table 2). Each compound in Table 1 was submitted and tested both as the anhydride and the corresponding diacid. We consistently observed no significant differences in binding affinity (within experimental error) for a given molecule in either form. We also observed that the anhydrides were easily hydrolyzed to diacids in the presence of water. In fact, endothall anhydride has been reported as a desiccant.[13] As a consequence of this, we suspected that the anhydrides opened readily during the enzyme assay. In an attempt to maneuver away from the anhydride, we converted it into a variety of functional groups. A number of amides, imides, esters and reduction products were synthesized. In general, we found that manipulation of the anhydride/diacid resulted in compounds which no longer inhibited the enzyme.

In conclusion, potent inhibitors of calcineurin, based on the known compound endothall, were synthesized. Ultimately, a 23 fold enhancement in binding, relative to endothall, was obtained. Improvements were made toward calcineurin inhibition while the effect of these ligands on the related enzyme PP1 remained constant. We accomplished this using protein structure-based tools and techniques. This work could serve as a stepping stone on the path to more potent and selective inhibitors of calcineurin.

Acknowledgment. We thank Dr. Peter S. Dragovich and Dr. Robert Babine for insightful design discussions, and Dr. Hans E. Parge for useful calcineurin structure function information.

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10. The PP2B assay was run as previously described,[23] with the omission of FKBP. The PP1 assay was run in the same fashion, with the following changes. The assay mixture contained 50 mM MOPS (pH 7.5), 10 mM dithiothreitol, 10 mM $MnCl_2$, 10 μM phosphorylase A, 25 nM rabbit recombinant PP1 (a generous gift fron Dr. Angus Nairn, Rockefeller Univ.) 10 μg/ml purine ribonucleoside phosphorylase, 200 μM methylthioguanosine[24] and 1% DMSO. Apparent Ki's were determined by analyzing the concentration dependence of inhibitors on the activity of PP2B or PP1, and fitting the data to a competitive tight binding inhibition equation.[25] Ki's were based on a single determination using multiple concentrations of inhibitor.
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19. A sample of endothall-5-carboxylic acid[13] was dissolved in DMSO at a concentration of 10 mM, mixed in a 6:1 molar ratio with a complex of CaN and FKBP12-FK506, and stored at 4 °C. Crystals of the CaN-FKBP12-FK506-ligand complex were obtained by vapor diffusion using hanging drops. The reservoir solution contained 10% monomethylether polyethylene glycol 2000, 1 mM $CaCl_2$, and 60 mM BME in 100 mM Bis Tris buffer at pH 6.5. Crystals with the morphology of tetragonal bipyramids formed in two to five days. X-ray diffraction data were collected at the Stanford Synchrotron Radiation Laboratory using a MAR image plate system from a single crystal flash-cooled (-181 °C) in 30% glycerol.. The overall R_{sym} was 0.079 for 127,332 observations of 19257 reflections collected to a resolution of 3.0 Å (99.5% of possible data). The space group was $P4_32_12$, with unit cell dimensions a = b = 105.0 Å, c = 164.9 Å. The 3.0 Å co-crystal structure did not allow unambiguous interpretation of the electron density, it did provided a calibration point for modeling of endothall analogs. To search for a binding mode consistent with the X-ray diffraction density, multiple docking simulations were performed using evolutionary programming search techniques.[26] One dominate binding mode was found. The ligand-protein complex was energy minimized with MacroModel substructure minimization protocols.[27] This study validated the modeling method and provided a reasonably oriented ligand for design of new analogs. All inhibitors were docked flexibly using the same methods. The 5-*endo* substituents were preferred, and both trans-cyclopropanes of compound **2j** appeared to fit equally well with slightly different bridging conformations.

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22. (a) Representative procedure for the synthesis of esters **1a-j**. **4-Phenylbutyric acid furan-3-ylmethyl ester** (**1d**): EDC·HCl (2.15 g, 11.21 mmol, 1.1 equiv) and DMAP (0.37 g, 3.06 mmol, 0.3 equiv) were added sequentially to a solution of 3-furanmethanol (1 g, 10.19 mmol, 1 equiv) and 4-phenylbutyric acid (1.84 g, 11.21 mmol, 1.1 equiv) in CH_2Cl_2 (25 mL) at 0 °C. The result was stirred at 23 °C for 3 h and then partitioned between water (100 mL) and EtOAc (2 x 100 mL). The crude product was purified by flash chromatography (10% EtOAc in hexanes) to provide ester **1d** (2.24 g, 90%) as a colorless oil: R*f* = 0.8 (30% EtOAc in hexanes); IR (film): 2938, 1732, 1504, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.93-2.07 (m, 2H), 2.35 (t, 2H, *J* = 7.5 Hz), 2.66 (t, 2H, *J* = 7.5 Hz), 5.00 (s, 2H), 6.44 (s, 1H), 7.16-7.49 (m, 7H). Anal. ($C_{15}H_{16}O_3$) C, H.
(b) Representative procedure for the synthesis of adducts **2a-j**. **4-Phenylbutyric acid 3,5-dioxo-4,10-dioxa-tricyclo[5.2.1.0 2,6]dec-8-ylmethyl ester** (**2d**). A solution of ester **1d** (0.44 g, 1.80 mmol, 1 equiv) and maleic anhydride (0.18 g, 1.80 mmol, 1 equiv) in anhydrous Et_2O was stirred at 23 °C for 24 h. The resulting white precipitate was filtered, washed with cold Et_2O and dried. To a solution of this material (0.54 g, 1.58 mmol) and DME (8 mL) in a small parr bottle was added 10% Pd/C (110 mg). This was hydrogenated at 50 psi for 2 h. The catalyst was filtered through celite and the filtrate was concentrated to provide pure adduct **2d** (0.51 g, 67%) as a white solid: mp 95-96 °C. IR (KBr): 3027, 1732, 1603, 1454 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.15-1.25 (m, 2H), 1.92-2.17 (m, 3H), 2.33-2.39 (m, 2H), 2.64-2.70 (m, 2H), 3.11 (d, 1H, *J* = 7.5 Hz), 3.47 (d, 1H, *J* = 7.5 Hz), 3.94 (dd, 1H, *J* = 11.8, 9.7 Hz), 4.22 (dd, 1H, *J* = 11.8, 5.9 Hz), 4.94-5.00 (m, 2H), 7.17-7.32 (m, 5H). Anal. ($C_{19}H_{20}O_6$) C, H.

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