Letters

Synthesis and Biological Evaluation of Non-Peptidic Cyclophilin Ligands

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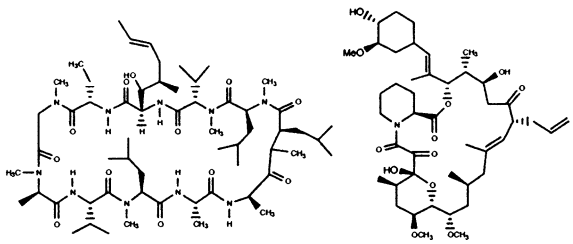
Received September 18, 2002

Abstract: Peptidylprolyl isomerase cyclophilins play critical roles in a variety of biological processes. Recent findings that cyclophilins are present at high levels in the CNS and that cyclosporin A may possess neuroprotective/neurotrophic effects have prompted us to search for nonimmunosuppressant small molecule cyclophilin ligands. To this end, we report the lead identification through "virtual screening" and the synthesis of our first series of non-peptidic cyclophilin ligands, along with the preliminary biological results.

Introduction. Immunophilins are binding proteins for the clinically important immunosuppressant drugs cyclosporin A (CsA), FK506, and rapamycin (Figure 1).[1,2] They are designated as cyclophilin A (CyPA), which binds CsA, and the 12 kDa FK506 binding protein (FKBP12), which binds FK506 and rapamycin. The drug−immunophilin complexes bind to target proteins: calcium/calmodulin dependent protein phosphatase calcineurin for CsA/CyPA and FK506/FKBP12; serine/threonine kinase FRAP for rapamycin/FKBP12.[3,4] CyPA and FKBP12 also possess enzymatic (peptidylprolyl isomerase, PPIases, or rotamase) activity,[5] although this activity is not directly related to the immunosuppressant effects elicited by the drug−immunophilin complexes. Although the immunosuppressant drugs shown in Figure 1 are potent and selective inhibitors of the PPIase activity of their cognate immunophilins, it is the interaction of the drug−immunophilin complexes that is responsible for suppression of the immune response.[6]

FKBP12 has been found to be present in nearly 50-fold higher concentration in the central nervous system compared to the immune system.[7] It was subsequently found that FK506 mimicked the effects of trophic factors such as nerve growth factor in vitro and in vivo.[8−10] We have previously demonstrated that analogues of FK506, which were modified in their "effector" domains so that they no longer interacted with their secondary protein target, retained the neurotrophic abilities of the parent drugs while failing to induce immune system suppression. Subsequently, small-molecule acyclic mimetics of the FKBP binding domain portion of FK506 were prepared and shown to be potent, systemically active neurotrophic drugs.[11−13] Consideration of the structure of the complexes of these molecules with FKBP12 led to the derivation of a number of patentably distinct, potent FKBP ligands.



Cyclosporin A FK506

Figure 1. Immunosuppressant drugs.

Similar to FKBP12, CyPA is present at high levels in the brain.[14] Increasing evidence suggests the therapeutic utility for cyclophilin ligands in treating neurological disorders.[15−17] We are following a similar path in order to exploit cyclophilin as a new target for neurological drug design. We demonstrated that non-immunosuppressant analogues of cyclosporin A, modified in the "effector" domain, are potent nerve growth agents. For example, MeVal−4-CsA and MeAbu−4-CsA are two cyclosporin analogues with modifications in the fourth amino acid position that are extremely potent CyPA inhibitors (K_i = 6 and 7 nM, respectively) and retain the in vitro neurotrophic activity of cyclosporin A but are nonimmunosuppressive.[18] To this end, we report the lead identification through "virtual screening" and the synthesis of our first series of non-peptidic cyclophilin ligands, along with their preliminary in vitro neuroprotective/neurotrophic activity.

Discovery of Lead Structures. The problem of designing small-molecule cyclophilin ligands is challenging because of the lack of appropriate non-peptidic ligands for cyclophilin to serve as starting points for rational drug design, as was the case for FKBP12. CsA and its assorted analogues are all large cyclic peptides that are complex and labor-intensive to synthesize. As in the case of our FKBP12 inhibitor work, a structure-based rational approach has been implemented that utilizes the tools of structural biology and computational chemistry in conjunction with combinatorial chemistry and synthetic organic chemistry. Key pieces of information to begin the work were (a) the crystal structure of CsA bound to CyPA (as well as several dipeptides bound to CyPA) and (b) the knowledge of the active site of cyclophilin gained from these structures.[19−21] This information was utilized in a "virtual screening" approach for discovery of initial lead structures.

Initially, the crystal structures of cyclosporin, cyclosporin analogues, and various dipeptides, complexed with CyPA were analyzed to derive a pharmacophore model for potential cyclophilin ligands. The fundamental pharmacophore consists of a hydrophobic portion that

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10.1021/jm020409u CCC: $25.00
Published on Web 02/25/2003

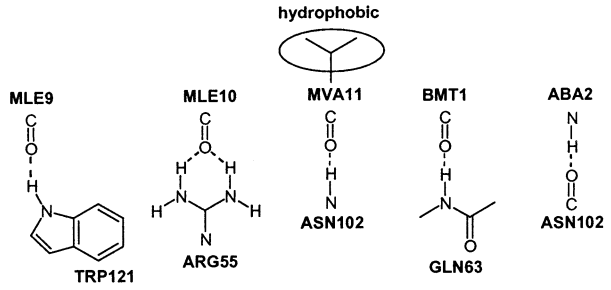


Figure 2. Pharmacophore from the cyclosporin−cyclophilin complex.

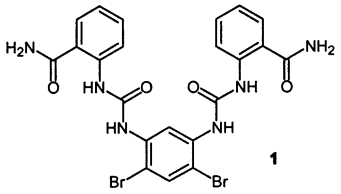


Figure 3. "Lead" structure identified by virtual screening.

binds to the central (proline-binding) pocket, and a group of hydrogen bonding centers, including one acceptor atom and four donor atoms, that form five hydrogen bonds with CsA. The residues involved are Trp-121, Arg-55, Asn-102 (which forms two H bonds), and Gln-63 (Figure 2).

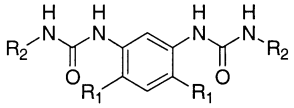
Three databases were screened for possible cyclophilin ligands: Available Chemicals Database, World Drug Index, and Chapman-Hall Dictionary of Organic Compounds. To keep computational time within reason, abstract representation of the cyclophilin binding domain was used, based on the above pharmacophoric analysis. Volume spheres were used to define the hydrogen bonding points, as well as volume-excluded regions of the pocket (to define roughly the areas of the pocket into which ligands are not physically capable of penetrating).

Before the screenings of these compound databases, filtering was done to remove undesirable molecules, i.e., those with molecular weights over 700 or those containing reactive functional groups, etc. The remaining compounds from each database were converted to 3D structures using the program Concord software[22] and were stored in a Unity[23] database. A Unity 3D pharmacophore search of these databases was performed using our cyclophilin pharmacophore as a query. Compounds that had a hydrophobic group capable of fitting into the cyclophilin pocket while simultaneously forming at least three of the five hydrogen bonds formed by CsA were scored as hits.

Inspection of the hit lists and their docked structures fitted onto the cyclophilin provided a basis for choosing potential cyclophilin ligands. Compound **1** was identified as a "lead" (Figure 3) and subsequently shown to be an inhibitor of cyclophilin rotamase activity with an IC_{50} of 6 μM. Therefore, this compound was selected to be a starting point for the initial SAR.

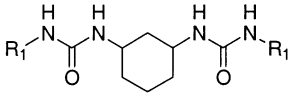
Chemistry and Biology. Compounds in Tables 1−4 were prepared according to Schemes 1 and 2, which are available in Supporting Information. In vitro biological assays, including inhibition of the rotamase activity of CyPA, protection of spinal cord neurons against excitotoxic cell death, and stimulation of neurite outgrowth

Table 1. Symmetrical 1,3-Phenyl Bis-ureas



compd	R_1	R_2	% inhibition at 10 μM	IC_{50} (nM)
1	Br	2-carbamoylphenyl		5900
2	Br	4-dimethylaminophenyl	98	2900
3	Br	4-iodophenyl	100	590
4		benzyl	19	
5		*n*-butyl	23	
6		2-pyridinyl	0	
7		3-isoxazole	93	4000
8		naphthalen-1-ylmethyl	100	1920
9		2,2-diphenyl-ethyl	100	4850
10		3-cyanophenyl	38	
11		3-methoxyphenyl	8	
12		2,4-dimethylphenyl	100	8500
13		2,4-dibromophenyl	100	6200
14		3,5-dichlorophenyl	100	2950

Table 2. Symmetrical 1,3-Cyclohexyl Bis-ureas[a]



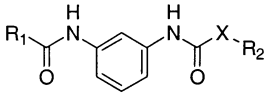
compd	R_1	% inhibition at 10 μM	IC_{50} (nM)
15	*tert*-butyl	7	
16	cyclohexyl	24	
17	1-admantyl	28	
18	benzyl	11	
19	4-chlorophenyl	32	
20	3-cyanophenyl	22	
21	3-methoxyphenyl	100	9500
22	2,4-dimethylphenyl	40	
23	naphthalen-1-yl	100	4550
24	2,4-dibromophenyl	100	5090
25	3,5-dichlorophenyl	100	4200

[a] Compounds in Table 2 consist of a mixture of cis and trans isomers.

from sensory neuronal cultures, were performed as described previously.[24−26] Values of rotamase inhibition IC_{50} are the mean of at least three independent estimations. Standard deviations were generally less than 15%.

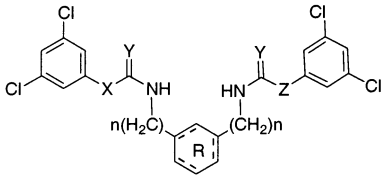
Results and Discussion. A large number of 1,3-bis-substituted aryl structures similar to compound **1** were synthesized and evaluated for the structure−activity relationship (Tables 1−4). Percent inhibition at 10 μM or IC_{50} values of synthesized compounds against CyPA were obtained and used as measures of relative ligand binding affinities. Examples of variations in the initial hit structure include different substitutions on the aryl portions, changing the central core, and changing one or both urea linkers into amide or thiourea linkers. Generally, alkyl substituents, even bulky (adamantyl) groups (compounds **5** and **15**−**17**), are not beneficial; however, introduction of large aromatic substituents (compounds **8**−**9**, **23**, and **27**−**29**) is favorable. Halogen (compound **3**) or multiple-halogen (compounds **13**−**14**, **26**, **36**−**37**, and **41**) substitutions on the aryl rings substantially increase potency. Neither electron-withdrawing groups nor electron-donating groups at the meta position of the aryl rings affect the activity (compounds **10**, **11**, and **20**). Replacement of the aryl group with the basic pyridinyl group cannot be tolerated (compounds **6** and **32**), leading to a complete loss of

Table 3. Symmetrical/Unsymmetrical 1,3-Phenyl Bis-amides/Amido-ureas



compd	X	R_1	R_2	% inhibition at 10 μM	IC_{50} (nM)
26		3,5-dichlorophenyl	3,5-dichlorophenyl		930
27		3,5-dichlorophenyl	naphthalen-1-ylmethyl		1120
28		3,5-dichlorophenyl	naphthalen-2-ylmethyl		880
29		3,5-dichlorophenyl	2,2-diphenylethyl		870
30		3,5-dichlorophenyl	benzyl	44	
31		5-chloro-2-methylsulfanylphenyl	5-chloro-2-methylsulfanylphenyl		1020
32		3-pyridinyl	3-pyridinyl	6	
33	NH	3,5-dichlorophenyl	phenyl		1180
34	NH	3,5-dichlorophenyl	4-trifluromethylphenyl		940
35	NH	3,5-dichlorophenyl	2-ethylphenyl		1410
36	NH	3,5-dichlorophenyl	3,5-dichrolophenyl		690
37	NH	3,5-dichlorophenyl	2,4-dibromophenyl		620
38	NH	3,5-dichlorophenyl	benzyl		3350
39	NH	3,5-dichlorophenyl	2-methylbenzyl		2120

Table 4. Comparison of Different Central Cores and "Arm" Linkers



compd	*n*	X	Y	Z	R	IC_{50} (nM)
14	0	NH	O	NH	phenyl	2950
40	1	NH	O	NH	phenyl	3950
25	0	NH	O	NH	cyclohexyl	4200
26	0		O		phenyl	930
36	0	NH	O		phenyl	690
41	0	NH	S	NH	phenyl	480

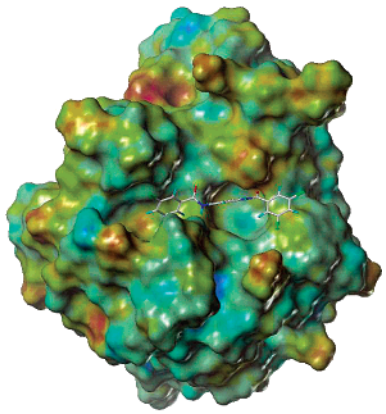


Figure 4. Predicted binding mode of **26** when bound to CyPA.

activity. On the other hand, the use of the isoxazole group is apparently beneficial (compound **7**). In the studied set of the central core, the potency is increased in the order phenyl > benzyl ≥ cyclohexyl ring (compounds **14** > **40** ≥ **25**). Among different linkages, the potency increases in the order bis-thiourea ≥ amido-urea > bis-amide > bis-urea (compounds **41** ≥ **36** > **26** > **14**).

To better understand the interaction between ligands and protein, the molecular docking program FLEXX[27] was utilized to dock the synthesized compounds onto the binding site of CyPA. The predicted binding mode for a representative compound **26** is shown in Figure 4. This model puts the central benzene core into the prolyl binding pocket. One of dichlorophenyl groups lies on an exposed hydrophobic surface of cyclophilin that is utilized by CsA, while the adjacent carbonyl of compound **26** interacts with the Arg55 side chain. The other dichlorophenyl group lies on a small ridge between the prolyl pocket and the second neighboring pocket. The amide linker interacts with CyPA, similar to the ProX dipeptides that have been crystallized with CyPA.[21] It should be pointed out that the second neighboring pocket is not utilized by CsA.

Like FKBPs, cyclophilins are also concentrated in the brain. Within the brain, CyPA is also highly colocalized with calcineurin, as is FKBP12. There are comparable levels of FKBP12 and CyPA in the hippocampal formation, cerebral cortex, hypothalamus, and cerebellum. Compared to CyPA, there are elevated levels of FKBP12 in the caudate, putamen, nucleus accumbans, globus pallidus, and olfactory tubercle. There are higher levels of CyPA in the thalamic nuclei, olfactory bulb, substantia nigra, pars compacta, raphe nucleus, and spinal cord. While there are regions of overlap and preferential expression of FKBP12 and CyPA, in general, distributions of immunophilins complement one another.

Owing to the high concentration of cyclophilins in spinal cord and motor neurons, we have evaluated a number of cyclophilin ligands for their protective effects against excitotoxic cell death in spinal cord cultures. Briefly, treatment of the spinal motor neurons in the culture with the glutamate reuptake inhibitor threo-hydroxyaspartate (THA) leads to 50−60% cell death. A number of cyclophilin ligands, such as **7**, **9**, **14**, and **31**, significantly protected these motor neurons from death at a screening dose of 10 μM, as shown in Figure 5.

To test the potential of cyclophilin ligands for neurotrophic effects, we applied compound **3** to adult dorsal root ganglion explant cultures at a screening dose of 1.0 μM. Compound **3** promoted the increased length of neurites after 48 h of drug treatment, as shown in Figure 6. The effects were clearly expressed in the increase of the percentage of longer neurites, as well as in the increase of the overall length of the processes. A limited dose−response analysis for compound **3** demonstrated increased neurite outgrowth at 10 nM concentration, with an EC_{50} between 100 and 1000 nM

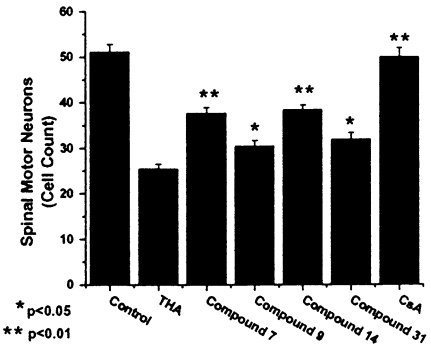


Figure 5. Cyclophilin ligands protect spinal cord neurons against excitotoxic cell death.

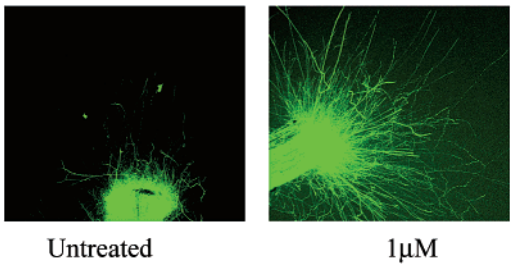


Figure 6. Photomicrographs of neurite outgrowth promoted by compound **3**.

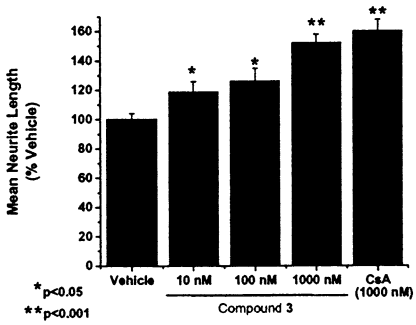


Figure 7. Dose−response curve of compound **3** in promoting neurite outgrowth.

(Figure 7). The affinity of this ligand for CyPA is 590 nM, which is in good agreement with the neurotrophic EC_{50}.

Conclusions. We have successfully identified, through "virtual screening" and SAR, novel non-peptidic cyclophilin ligands with submicromolar IC_{50}. Preliminary biological experiments have shown cyclophilin ligands to protect motor neurons from excitotoxic cell death and to stimulate neurite outgrowth from sensory neuronal cultures. Therefore, cyclophilin ligands may be useful as therapeutic agents targeting CNS diseases.

Supporting Information Available: Scheme 1 and 2 and general synthetic procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM020409U