Structure-Based Design, Synthesis, and Biological Evaluation of Novel Inhibitors of Human Cyclophilin A

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Cyclophilin A is involved in many cellular processes, including protein folding and intracellular transports. Because cyclophilin A has been shown to interact with HIV-1 gag proteins and to enhance the viral infectivity, nonimmunosuppressive cyclophilin A ligands may represent a new class of therapeutic agents against HIV. Here, we report a virtual screening using structure- and pharmacophore-based design to identify original nonpeptidic cyclophilin ligands. Following a lead identification of compounds **1** [1-(3-benzyloxypyridin-2-yl)-3-(3-chlorophenyl)urea] and **2** [1-(3-benzyloxypyridin-2-yl)-3-(3-trifluoromethylphenyl)urea] (IC_{50} = 0.3 μM), a series of molecules were synthesized from a diarylurea scaffold and evaluated for their in vitro ability to inhibit the cis−trans isomerase activity of cyclophilin A. Molecular modifications provided several more potent compounds, in particular analogues **4d** and **4i** with IC_{50} of 14 and 20 nM, respectively. Then, we evaluated the effect of analogues **1** and **2** on HIV virion infectivity in both immortalized and primary cells. Both **1** and **2** reduced virion infectivity in the replication-defective one-round infection assay, but only **1** impaired wild-type HIV infection in human peripheral blood mononuclear cells.

Introduction

The immunophilins are cytosolic enzymes that have been observed abundantly and ubiquitously in a wide range of tissue types and organisms.[1,2] They are characterized by the ability to catalyze the cis−trans isomerization of peptidylprolyl bonds[3] (PPIases), which was identified as the rate-limiting step in protein folding.[4,5] Concurrently to their function in protein maturation, immunophilins seems to play a key role in fundamental cellular processes, as they either stabilize or destabilize a protein−protein complex (immunosuppression, calcium channels,[6] steroid receptors,[7] and tyrosine kinase receptors[8]). Originally identified as the cellular targets of immunosuppressant drugs, the immunophilins are represented by two classes: FKBPs (FK506 binding proteins) and cyclophilins (Cyps) (reviewed in ref 9). Human cyclophilin A (CypA) was first described as the binding partner of cyclosporin A[10] (CsA), an immunosuppressive cyclic undecapeptide widely used to prevent organ rejection after transplant operations. CsA binds to the PPIase site on CypA, blocking its ability to direct cis−trans isomerization. However, the immunosuppressive role of CsA is not related to the inhibition of PPIase activity. Instead, the high-affinity CsA/CypA complex binds to calcineurin and inhibits its phosphatase activity, which in turn suppresses T cell activation.[11]

Recently, new potent applications for nonimmunosuppressive analogues of CsA have been reported. CypA is implicated in the formation of the viral capsid of HIV-1 through a binding interaction with the HIV-1 Gag precursor polyprotein.[12] Thus, nonimmunosuppressant cyclosporin A analogue blocking the PPIases activity was identified as potent inhibitors of HIV-1 replication.[13] Also, both immunosuppressive and nonimmunosupressive agents have demonstrated potent neuroprotective and neurotrophic effects in several animal models; however, the mechanism remains to be elucidated.[14,15] Interestingly, Wu et al. identified a set of nonpeptidic cyclophilin ligands with preliminary in vitro neuroprotective/neurotrophic activity.[16] All these studies suggest a potential therapeutic value for peptidic and nonpeptidic cyclophilin ligands as novel anti-retroviral drugs or in treating neurological disorders.

Herein, using a crystal structure of CsA in complex with CypA (pdb code 1CWA), a virtual screening approach was performed to identify novel inhibitors that can interfere with the active site of cyclophilin. Following database docking, commercially available compounds were selected and tested for inhibitory activity on cyclophilin. Then, based on the most potent new inhibitors, a set of nonpeptidic cyclophilin ligands was synthesized and anti-HIV activity was evaluated.

Results and Discussion

Structure-Based Design and Virtual Screening. To identify the inhibitory molecules of CypA, a structure-based approach was used for selecting the initial compound library designed for virtual screening. Several crystal structures of cyclosporin, cyclosporin analogues, and various dipeptides, complexed with CypA, are available in the Protein Data Bank. These structures determined by X-ray crystallography or NMR were analyzed to derive a pharmacophore model for potential cyclophilin ligands. Cyclophilin A adopts an eight-stranded antiparallel P-barrel structure in the CypA/CsA complex[17−19] CsA and other substrates bind a common relatively hydrophobic groove formed by 11−13 residues via hydrogen bonds and polar and hydrophobic contacts. On the basis of X-ray crystal structures, the most important residues of the active site were identified by site-directed mutagenesis (Arg55, Phe60, Phe113, and His126).[20]

As shown on Figure 1, analysis of the contact surface of the CypA/CsA complex (pdb code 1CWA) reveals that side chain of the MeVal11 of CsA is buried in a left hydrophobic pocket. By contrast, the side chain of the Abu2 of CsA lies in another cavity, the Abu pocket. Localized between these two residues,

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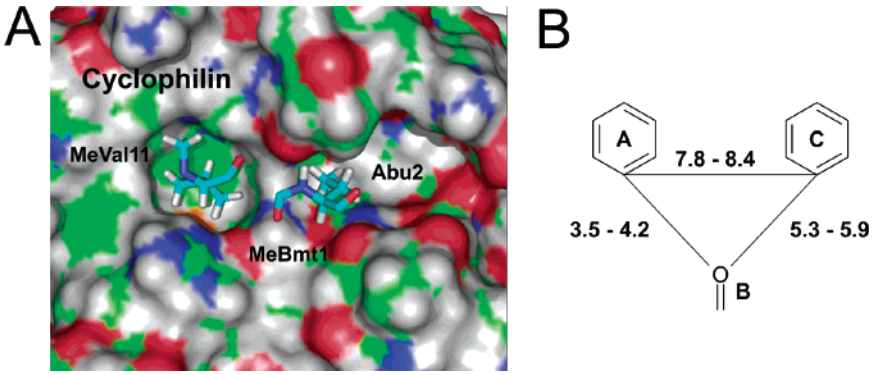


Figure 1. (A) The conformation of residues Abu2 and MeVal11 and the carbonyl oxygen of MeBmt1 of CsA in the CypA/CsA monomeric X-ray crystal structure is shown (pdb code 1CWA). The left hydrophobic pocket of CypA is filled with the MeVa11 side chain and the Abu2 is positioned at the beginning of the right pocket−Abu pocket. This image was generated and rendered with Pymol. The CypA surface was colored by atom type. (B) Schematic diagram outlining the ideal properties of a CsA pharmacophore. Numbers represent distances in angstroms.

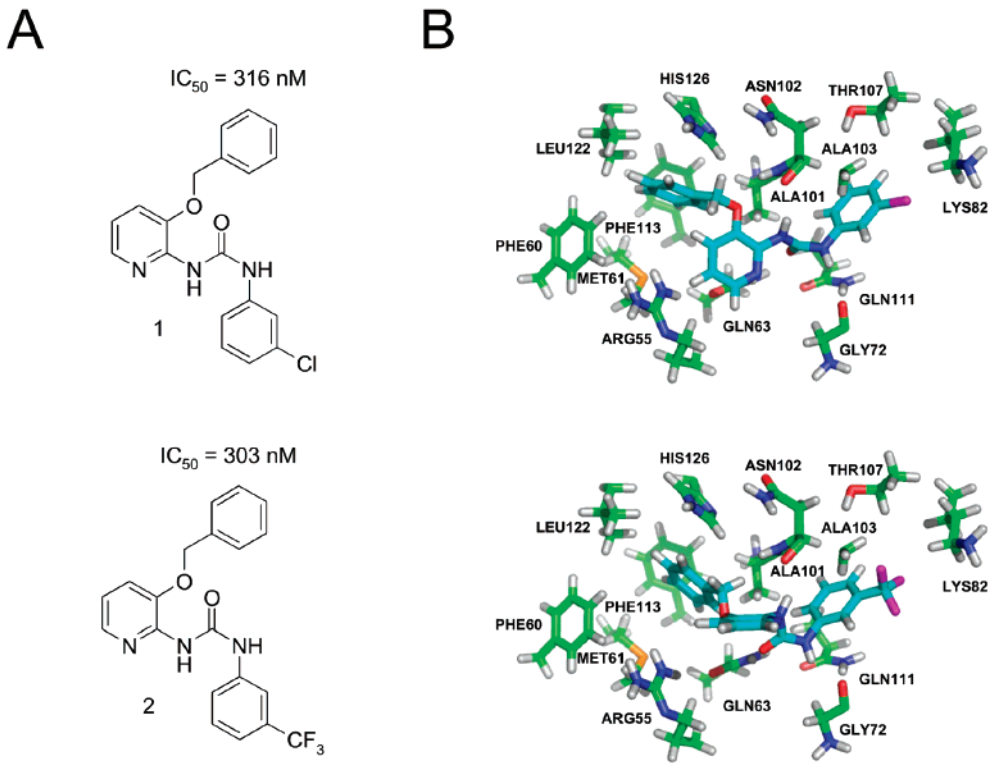


Figure 2. (A) Lead compounds **1** and **2** identified by virtual screening. (B) Putative binding mode of compounds **1** (upper, FlexX pose) and **2** (lower, Surflex pose) in the active site of human cyclophilin A. Possibilities of interaction with the surrounding amino acids include three hydrogen bonds between the urea moiety and Gln63, Gly72, and Asn102 as well as several hydrophobic contacts.

the carbonyl oxygen of MeBmt1 is at hydrogen-bond distance to the Nϵ of Gln63. On the basis of information derived from the cocrystal structure, three groups were chosen for the development of the pharmacophore (Figure 1, panel B). For two pharmacophoric points, a phenyl group (A, C) was selected to mimic and potentially reinforce hydrophobic contacts of MeVal11 and Abu2 with CypA. At first sight, the Abu pocket of CypA appears to be a good location to design high-affinity analogues with additional contacts. However, as illustrated by Mikol et al.,[21] three well-ordered water molecules that are parts of the crystal structure could interfere during the binding. By consequence, as a good compromise we decided to replace the Abu side chain with an aromatic fragment not larger than a phenyl group. Carbonyl oxygen (B) was kept for the central pharmacophoric point. Then, the distances between carbonyl oxygen and Cβ of the two side chains were measured and added to the pharmacophoric constraints. All residues that are within 10 Å from the Cβ of MeVal11 in the CypA structure were selected and used to define the binding site in our structure-based computer screening.

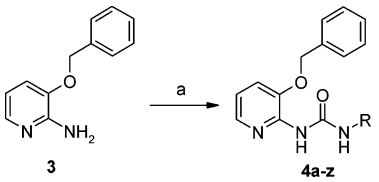
Using the MDL ISIS/Base software of the ACD-3D (Available Chemicals Directory, which contains associated 3D data, Release ACD 2004−2, 296 387 compounds in total), a 3D database search was performed to identify compounds that map all the pharmacophoric features. A subset of 3129 molecules having molecular weights up to 650 Da was so defined. These molecules were docked and evaluated by the FlexX program[22] and then co-evaluated by using the X-score program.[23] Molecules with FlexX energy scores from −25 to −40 or with X-score energy scores from 5 to 7 were visually analyzed for their associations with the left hydrophobic pocket of cyclophilin and the capability to form hydrogen bond interactions through their carbonyl oxygen. At the final stage, molecules that show promising docking affinities in at least one of the scoring method and a good binding mode in accordance with our pharmacophoric definition were purchased. Taking into account the

Table 1. Most Significant FlexX and Xscore Scores of Selected Compounds[a]

compd	IC_{50} (nM)	FlexX	Xscore	compd	IC_{50} (nM)	FlexX	Xscore
1	316	−26.03	6.22	**4d**	14	−34.10	6.27
2	303	−25.89	6.31	**4i**	20	−24.75	5.64
4a	754	−23.57	5.96	**9c**	140	amb	
4s	nd	−27.59	6.29	**11**	60	amb	
4o	592	−25.98	6.51	**12**	320	amb	
4c	60	amb		**15**	nd	not docked	
4h	951	−22.90	5.64	**16a**	nd	−26.05	6.49
9b	71	amb		**16b**	4100	−18.66	5.38
7b	528	−35.15	6.64	**17**	nd	−23.10	5.92
6b	nd	−33.00	6.21	**18**	nd	not docked	
4y	nd	not docked		**19**	nd	not docked	

[a] Compounds were docked/scored with FlexX and then rescored using Xscore. For FlexX, more-negative scores are better. The Surflex score for compounds **1** and **2** was 4.96 and 4.92, respectively. amb = abnormal mode of binding; nd = not determined.

Scheme 1[a]



[a] Reagent: (a) R−N=C=O/THF.

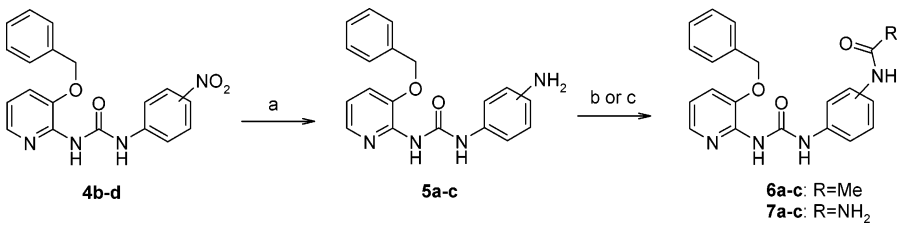
availability of the compounds, we focused on a set of 31 chemicals for further characterization against the cis−trans isomerase of cyclophilin A. Among them at 10 μM, five compounds turned out to inhibit more than 80% of the isomerase activity of cyclophilin A and four compounds were less potent with a 40% inhibition. The IC_{50} values for the former five compounds were experimentally determined as lying between 0.3 and 5 μM (data not shown). Compounds **1** [1-(3-benzyloxypyridin-2-yl)-3-(3-chlorophenyl)urea from Menai] and **2** [1-(3-benzyloxypyridin-2-yl)-3-(3-trifluoromethylphenyl)urea from Menai] were stronger inhibitors of the isomerase activity with an IC_{50} of around 0.3 μM and 100% inhibition at 10 μM. As shown in Figure 2 (panel A), **1** and **2** present a diarylurea scaffold that is appropriate for rapid molecular optimization, and thus, it was selected for further molecular modifications.

The putative interaction mode of compounds **1** and **2** with CypA was further docked and evaluated with a second docking program, Surflex.[24] As for FlexX, no water molecules were present during the virtual screening calculations. Compounds **1** and **2** possessed similar FlexX, X-score, and Surflex energy scores (∼26, ∼6, and ∼5, respectively) but were only top-ranked by Surflex (Table 1). They appeared within the active site in a comparable fashion with only small variations in distances to active site residues with the benzyl group buried into the left hydrophobic pocket and the phenyl ring positioned inside the Abu pocket as shown in Figure 2, panel B (compound **1**, FlexX pose; compound **2**, Surflex pose). The docked structures of 1 and 2 were analyzed to suggest how the diarylurea scaffold may be expanded and to further rationalize the structure−activity relationship. In comparison with CsA, compounds **1** and **2** retain two hydrogen bonds with critical residues Gln63 and Asn102 of CypA and present an additional bond with Gly72, one nitrogen atom of the urea moiety is hydrogen bonded to the carbonyl oxygen atom of Asn102, the carbonyl oxygen atom of the urea function is hydrogen bonded to the Nϵ atom of Gln63, and the second nitrogen atom is bonded to the carbonyl oxygen atom of Gly72. Furthermore, most of the hydrophobic interactions between CypA and CsA were conserved within the docking mode of compounds **1** and **2**. Thus, Arg55, Phe60, Ala101, Ala103, Phe113, Leu122, and His126 were found to form hydrophobic contacts with molecule **1** and **2**, whatever the docking poses proposed by FlexX and Surflex. Interestingly, as illustrated in Figure 2 (panel B), the 3-chlorophenyl and the 3-trifluoromethylphenyl group of **1** and **2**, respectively, were found to be directed toward a hydrophilic region of the Abu pocket composed by residues Gly72-Thr73-Gly74, Thr107-Asn108-Gly109-Ser110-Qln111, and Lys82. As described by X-ray studies[21] of several cyclophilin−cyclosporin complexes, this region is normally occupied by three to four water molecules, which form hydrogen bonds with hydrophilic residues.

Chemistry and Structure−Activity Relationships. According to the docking study between the CypA structure and **1** or **2**, the 3-benzyloxypyridine group was found to fulfill the left pocket and the monosubstituted phenyl group positioned toward the Abu pocket. Thus at a first step, we searched for substituents that could afford additional contacts with the beginning or the hydrophilic region of the Abu pocket, leaving the rest of the molecule intact. Except for **4j**, bisubstituted ureas **4a**−**z** were prepared by the condensation of 2-amino-3-benzyloxypyridine (**3**) with aryl isocyanate in THF under reflux (Scheme 1) in good to excellent yields. **4j** was obtained by selective reaction of the amino group of **3** with the isocyanate function of 4-(chlorosulfonyl)phenyl isocyanate at room temperature.[25] Reduction[26] of compounds **4b**−**d** with $SnCl_2$ in EtOH under reflux afforded amines **5a**−**c**. The amines **5a**−**c** were acylated with acetic anhydride to obtain the acetamide **6a**−**c** or converted to ureas **7a**−**c** upon the action of potassium cyanate[27] (Scheme 2). Hydrolysis of **4e**−**f** with H_2O_2[28] under basic conditions afforded amides **8a,b** (Scheme 3). Saponification of ethyl ester **4g**−**i** with NaOH gave carboxylic acids **9a**−**c** and ethyl ester **4h** was converted to methyl ester **10a** upon the action of sodium methanolate in MeOH (Scheme 4). Reactions of **4j** with aqueous NH_3 or aqueous $MeNH_2$ in THF gave the corresponding sulfonamide **11** and **12** (Scheme 5). Reactions of **4k** with aqueous NH_3 in THF gave a mixture of two compounds, **13** and **14**, which were separated by chromatography on silica gel (Scheme 6).

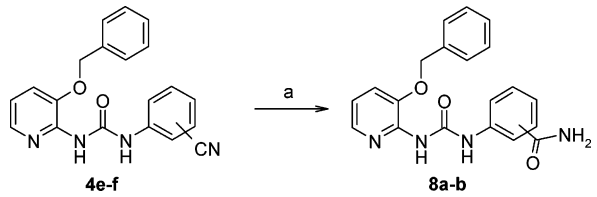
Then, we evaluated the influence of the 3-benzyl group. Phenol derivative **15** was obtained through catalytic hydrogena-

Scheme 2[a]



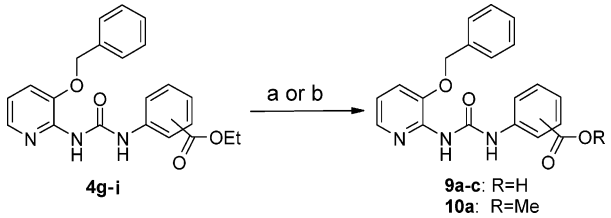
[a] Reagents: (a) $SnCl_2$/EtOH/reflux; (b) Ac_2O/THF; (c) KOCN/H_2O/AcOH/THF.

Scheme 3[a]



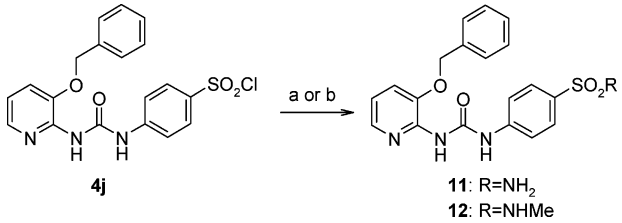
[a] Reagents: (a) $H_2O_2/Na_2CO_3/H_2O/THF/DMF$.

Scheme 4[a]



[a] Reagents: (a) $NaOH/H_2O/THF$; (b) NaOMe/MeOH.

Scheme 5[a]



[a] Reagents: (a) aq $NH_3/H_2O/THF$; (b) aq $MeNH_2/H_2O/THF$.

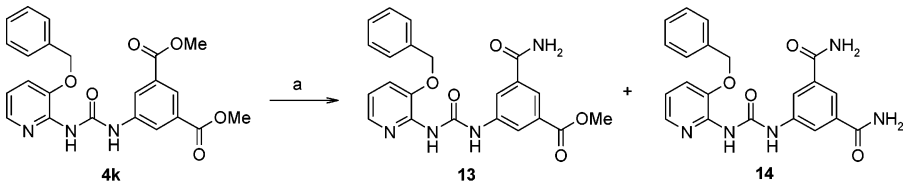
tion of **4a** over Pd/C in EtOH. Urea **16a,b** were prepared from **15**; thus, treatment of a solution of **15** in dry THF with NaH, followed by addition of alkylating agents at room temperature, gave the corresponding urea **16a,b** in good yields (Scheme 7). At last, we prepared three compounds, **17**−**19**, to confirm the importance of the urea moiety. Thiourea **17** was prepared by condensation of **3** with phenyl thioisocyanate in THF under reflux. Reaction of **3** with benzoyl chloride and benzenesulfonyl chloride gave the corresponding amide **18** and sulfonamide **19** in good yields (Scheme 8). All the chemical compounds were characterized for their inhibitory potency of the cyclophilin isomerase activity in a typical in vitro test.[29]

To improve the activity of meta-substituted compounds **1** and **2**, our initial focus was to explore the Abu pocket accessed by the phenyl ring. As indicated in Table 2, none of the ortho-substituted derivates showed better activity than the two lead compounds **1** or **2**. Shifting the *m*-Cl group (**1**, IC_{50} = 316 nM) to the *o*-position (**4o**, IC_{50} = 592 nM) resulted in reduction of the IC_{50} value by a factor of 1.5−2, while removing the Cl group (**4a**, IC_{50} = 754 nM) reduces the IC_{50} value by a factor of 2.5. It was found that introduction of an aliphatic chain or an aromatic ring resulted in a drastic loss of activity (**4l**, **4s**). Substitutions with more polar groups conduct to attenuated activity or no activity at all. For example, compound **9a** with a carboxylic acid function was completely inactive, whereas compound **4g** with an ethyl ester showed an IC_{50} of 880 nM. These results suggest that ortho substitutions on the phenyl ring might be unsuitable for compounds with an enhanced activity and that additional contacts have to be found deeper inside the Abu pocket.

Subsequently, we studied the effect of substituting the meta position of the phenyl ring (Table 2). For meta-substituted derivates, incorporation of an aliphatic chain (**4m**), an electron-withdrawing group such as a bromide (**4q**), a nitrile (**4e**), or an electron-donating group such as an acetamide moiety (**6b**) and, to a lesser extent, an urea group (**7b**) resulted in a significant decrease of the inhibition at 10 μM. Five other electron-withdrawing groups were also tested. Among them, three derivates (**4h**, **10a**, **8a**) showed less potent activity when compared to the lead compound **1** with IC_{50} values from 361 to 951 nM. Interestingly, it is worthy of note that several compounds reinforce the putative mode of binding and the orientation of the phenyl ring toward a hydrophilic region of the Abu pocket. First, comparison of compounds **4h**, **10a**, and **9b** clearly indicates that hydrophobic contacts decrease the efficiency of a molecule (IC_{50}: ethyl ester **4h** < methyl ester **10a** < carboxylic acid **9b**). Then, **4c** and **9b**, which present an electron-withdrawing group that is able to form hydrogen bonds, provide much better analogues, with an IC_{50} value of 60 and 71 nM, respectively.

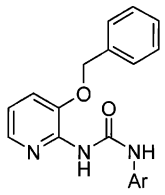
Various substitutions in the para position of the phenyl ring were explored (Table 3). It was observed that aromatic (**4y**) and electron-donating groups (**6c**, **7c**) led to a sharp drop in potency. Comparable to the SAR of meta-substituted derivates, *p*-phenyl analogues with an electron-withdrawing group led to various effects, from the most active compound in the series (**4d**, IC_{50} = 14 nM) to a compound with no activity at all (**4f**). In this position, it is interesting to note that hydrogen-bonding capability of substituents, preferably two double-bond oxygen atoms, plays an important role in the enhanced activity of the compound (**4d**, **4i**, **11**). On the another hand, di and trisubstituted phenyl analogues give poor results and that is certainly due to steric clashes with hydrophilic residues of the Abu pocket. Only one difluorinated compound is active (**4z**, IC_{50} = 458 nM), which presents a hydrogen-bond-acceptor capability (Table 3). The docking models revealed a good picture of the structure−activity relationship for the *p*-phenyl substituted analogues. Indeed, for instance, the nitrile group of the highly active molecule **4d** is localized near the coordinates normally occupied by two water molecules in several structures of cyclophilin (e.g., W6 and W7 of pdb crystal structure 1CWA). In the same way as these water molecules, the nitrile group of **4d** could be implicated in two hydrogen bonds with Thr107 and Gly109. Moreover, the presence of an ethyl ester group in the para position (**4i**) produced a very active compound, whereas the ortho and meta positions (**4g**, **10a**) conducted to a decrease in activity. Again, this can be explain by docking models that show

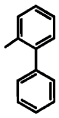
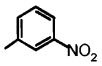
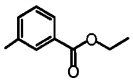
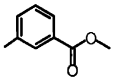
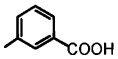
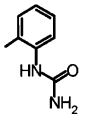
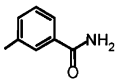
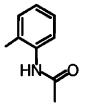
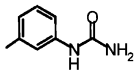
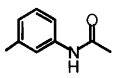
Scheme 6[a]



[a] Reagent: (a) aq $NH_3/H_2O/MeOH$.

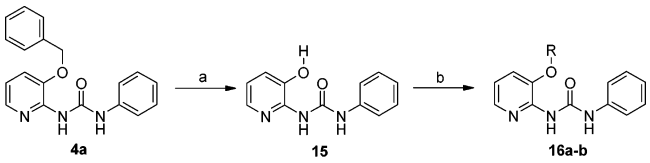
Table 2. In Vitro Inhibitory Activities of Diarylurea Analogues Substituted on the Ortho Position (left) and Meta Position (right) of the Phenyl Ring[b]



Compound	Ar	% inhibition 10 μM	IC_{50} (nM) (n)[a]	Compound	Ar	% inhibition 10 μM	IC_{50} (nM) (n)[a]
4a		100 (1)	754 (3)	**4m**		12 (2)	n.d.
4s		21 (2)	n.d.	**4q**		11 (2)	n.d.
4l		0 (1)	n.d.	**4e**		55 (1)	5500 (3)
4o		100 (1)	592 (3)	**4c**		100 (1)	60 (3)
4b		40 (2)	n.d.	**4h**		100 (1)	951 (3)
9a		25 (2)	n.d.	**10a**		95 (1)	748 (3)
4g		100 (1)	880 (3)	**9b**		100 (1)	71 (3)
7a		8 (2)	n.d.	**8a**		95 (1)	361 (3)
6a		35 (2)	n.d.	**7b**		89 (3)	528 (3)
				6b		52 (2)	n.d.

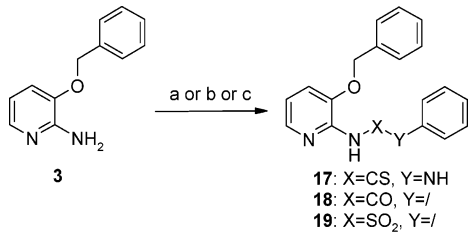
[a] Number of determinations. [b] Data of the percent inhibition at 10 μM are obtained from triplicate measurements. IC_{50} data represent mean values of three independent determinations and SD is typically lower than 8%. nd = not determined.

Scheme 7[a]



[a] Reagents: (a) Pd/C, H_2/EtOH; (b) NaH/THF then R−X.

Scheme 8[a]



[a] Reagents: (a) Ph−N═C═S/THF; (b) Ph-COCl/THF; (c) Ph-SO_2Cl/THF.

additional van der Waals contacts with the Lys82 side chain when the phenyl ring is para-substituted with an ethyl ester group.

We then investigated the influence of the benzyl group in the inhibitory potency of our analogues. By virtual docking, this benzyl group was found to sit in the hydrophobic left pocket that is normally occupied by the side chain of MeVal11 of CsA. Compounds **16a** and **15** with either a bulky aromatic group or no moiety present a sharp decrease in activity, whereas compound **16b** (IC_{50} = 4.100 nM) with an aliphatic group was less active than the parent compound **4a** (IC_{50} = 754 nM) (Table 4). Finally, to confirm the role of the urea moiety, which seems implicated in several hydrogen bonds with the protein, we replaced the central part of the molecule with a thiourea (**17**), an amide (**18**), and a sulfonamide (**19**) and found these compounds to be far less potent (**19**, 45% inhibition at 10 μM) or even inactive. These results are in total agreement with the binding model proposed for **1** and **2**, which shows that the urea moiety forms three hydrogen bonds with the protein.

Taken together, these results indicate that the pharmacophore used in this study represented an efficient scaffold to mimic critical interactions between a ligand and the human cyclophilin. Five compounds over 31 commercially chemicals initially tested were found to inhibit more than 80% of the cyclophilin activity. On the basis of the reported SARs of our 47 original compounds, we concluded that the two docking algorithms FlexX and Surflex were able to generate correctly oriented docked poses for molecules **1** and **2**. However, as is usually the case with virtual screening, it was not possible to differentiate between closely related active and inactive analogues (Table 1). The correlation between SARs and docking studies clearly reveals that intermolecular hydrogen bonding, between meta and para functional groups of the phenyl ring and residues localized into the Abu pocket of the cyclophilin, allowed an increase of the inhibitory potency of our nonpeptidic analogues of CsA. The hydrophilic part of the Abu pocket is normally stabilized via a hydrogen-bonding network occurring through three well-ordered water molecules.[21] Here, we proposed that phenyl-substituted groups with hydrogen-bonding capability either displace or replace at least one of these water molecules. However, in absence of X-ray analysis, the precise nature of residues implicated in the hydrogen-bonding network between a compound and the cyclophilin A remains to be defined. Indeed, X-ray structural experiments are under progress and will allow the design of new analogues with single-digit nanomolar range activity.

Anti-HIV-1 Activity. The ability of compounds **1** and **2** to interfere with the HIV-1 replication cycle was evaluated in a one-round infection assay, where infectious but nonreplicative virions harboring a luciferase reporter gene produced in 293T cells were used to infect target HOS-$CD4^+$-$CCR5^+$ cells. When 10 μM of CsA or compounds **1** and **2** was added to both producing and target cells, virions showed a dramatic decrease of infectivity of respectively 96%, 97%, and 62% (Figure 3A). This 96% decrease for CsA is slightly above the 85−88% generally described.[30,31] This could be due to different experimental systems. Interestingly, as previously described for CsA,[32] the decrease in infectivity is almost entirely due to CypA inhibition in virions producing cells, since virions produced in the presence of inhibitors showed a similar magnitude of decrease in infectivity while the target cells are cultured in the absence of inhibitors (Figure 3B). On the other hand, the treatment of inhibitors in target cells only had a modest impact on infectivity of control virions produced without inhibitors treatment (Figure 3C). This effect has recently been shown to be independent of the CypA−capside interaction; however, the mechanism still needs to be defined.[33,34]

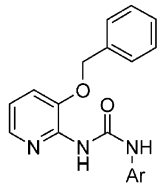
We next tested compounds **1** and **2** on the replication of the pNL4.3 strain of HIV-1 in human peripheral blood mononuclear cells (PBMC). We first tested that the compounds had no effect on cell viability and proliferation. In contrast to CsA, which interferes with T cell activation via the calcineurin pathway and blocks potential cell proliferation,[11,35] cells counts were comparable between different culture conditions with/without **1** or **2** inhibitors (data not shown). Interestingly, this result suggests that compounds **1** and **2** could be nonpeptidic cyclophilin inhibitors with nonimmunosuppressive activity. However, the nonimmunosuppressive property of these diarylurea analogues has to be confirmed with more accurate experiments such as their incapacity to inhibit the activation of specific transcription factors that regulate genes that are expressed early in T lymphocyte activation.

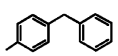
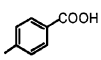
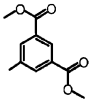
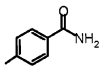
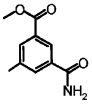
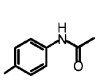
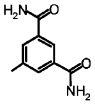
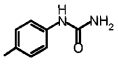
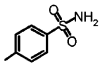
As the block of PBMC growth per se is deleterious on HIV production, the CsA inhibition of infectivity can be explained by two different mechanisms: interaction with CypA during virion production and inhibition of cell proliferation. We then decided to compare the efficiency of the two compounds to that of a potent anti-HIV drug without effect on cell proliferation, AZT, a reverse transcriptase inhibitor. As the first viral inoculum is produced without inhibitors, the chemical analogues will only act on subsequent viral replication cycles. AZT (50 μM) was then added 12 h after the initial infection in a control assay to allow the first round of infection and inhibit further replicative cycles. Compound **1** retained a dose dependent inhibitory effect on replicative virions, 60% of the AZT effect at 10 μM, while compound **2** had no effect at all on HIV-1 infectivity in these primary cells (Figure 4). The later was already much less efficient in the one-round infection assay, maybe due to poor membrane permeability. It is interesting to note that compound **1** at 1 μM is more efficient as the number of replication cycle increases (compare days 4 and 7 postinfection), which is consistent with previous reports.[36]

Conclusion

In summary, we employed a structure-based design to identify compounds able to interfere with the active site of cyclophilin. Five out of 31 selected compounds were found to induce an in vitro inhibition of the isomerase activity of cyclophilin A. On

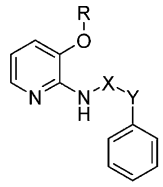
Table 3. In Vitro Inhibitory Activities of Diarylurea Analogues Substituted on the Para Position of the Phenyl Ring (left) and with Multiple Substitutions (right)[b]



Compound	Ar	% inhibition 10 μM	IC_{50} (nM) (n)[a]	Compound	Ar	% inhibition 10 μM	IC_{50} (nM) (n)[a]
4y		5 (1)	n.d.	**4z**		100 (1)	458 (3)
4x		100 (1)	640 (3)	**4r**		35 (2)	n.d.
4t		79 (2)	499 (3)	**4n**		63 (2)	n.d.
4f		49 (2)	n.d.	**4p**		74 (1)	n.d.
4d		100 (1)	14 (3)	**4u**		0 (2)	n.d.
4i		100 (1)	20 (3)	**4v**		29 (2)	n.d.
9c		98 (1)	140 (3)	**4k**		34 (1)	n.d.
8b		84 (1)	810 (3)	**13**		5 (1)	n.d.
6c		63 (1)	1437 (3)	**14**		19 (2)	n.d.
7c		44 (2)	n.d.	**4w**		12 (2)	n.d.
11		96 (1)	60 (3)				
12		97 (1)	320 (3)				

[a] Number of determinations. [b] Data of the percent inhibition at 10 μM are obtained from triplicate measurements. IC_{50} data represent mean values of three independent determinations and SD is typically lower than 8%. nd = not determined.

Table 4. In Vitro Inhibitory Activities of Compounds **15**, **16a**, and **16b** and Thiourea (**17**), Amide (**18**), and Sulfonamide (**19**) Analogues[b]



Compound	R	X Y	% inhibition 10 μM	IC_{50} (nM) (n)[a]
15	H	CO NH	10 (1)	n.d.
16a		CO NH	55 (2)	n.d.
16b		CO NH	95 (2)	4100.
17		CS NH	25 (2)	n.d.
18		CO /	22 (2)	n.d.
19		SO_2 /	45 (2)	n.d.

[a] Number of determinations. [b] Data of the percent inhibition at 10 μM are obtained from triplicate measurements. IC_{50} data represent mean values of three independent determinations and SD is typically lower than 8%. nd = not determined.

the basis of compounds **1** [1-(3-benzyloxypyridin-2-yl)-3-(3-chlorophenyl)urea] and **2** [1-(3-benzyloxypyridin-2-yl)-3-(3-trifluoromethylphenyl)urea], we developed a novel class of potent diarylurea inhibitors and designed compounds with enhanced activity (analogues **4d** and **4i** with IC_{50} of 14 and 20 nM, respectively). Then, we have shown that compounds **1** and **2** are potent inhibitors of the HIV-1 replication cycle on immortalized cells or human peripheral blood mononuclear cells. Therefore, these new nonpeptidic cyclophilin ligands might serve as novel leads for further pharmacological investigations as therapeutic agents against HIV-1.

Experimental Section

General Methods. All reagents and solvents were purchased from commercial sources and used without further purification. Melting points were determined on a Kofler block and are uncorrected. Mass spectra were acquired under fast atom bombardment (FAB) conditions using a JEOL JMS DX300, and samples were introduced on a matrix. Proton NMR spectra were collected on a Bruker 400 MHz spectrometer using tetramethylsilane (TMS) as internal standard, and chemical shift (δ) data are reported in parts per million (ppm) relative to internal standard TMS. Thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} plates from Macherey-Nagel and visualized by UV light. Flash chromatography was carried out using SDS silica gel 60 (35−70 mesh) with cyclohexane, ethyl acetate (EtOAc), and methanol (MeOH) as eluents with chromatographic solvent proportion expressed on a volume:volume basis. The reported chemical yields were not optimized.

Synthesis of Ureas 4a−z: General Procedure (except for 4j). 2-Amino-3-benzyloxypyridine **3** (1 equiv) was dissolved in dry THF (0.25 M). The aryl isocyanate (1.2 equiv) was added in one portion and the reaction mixture was heated under reflux. After the reaction was complete (TLC control), the reaction mixture was concentrated and Et_2O was added to precipitate the urea. The solid was filtrated, washed with a small amount of Et_2O, and dried under vacuum.

4-[3-(3-Benzyloxypyridin-2-yl)ureido]benzenesulfonyl Chloride (4j). 2-Amino-3-benzyloxypyridine **3** (200 mg, 1.0 mmol) was dissolved in 4 mL of dry THF and this solution was cooled with an ice bath. 4-Chlorosulfonylbenzene isocyanate (217 mg, 1.0 mmol) was added drop by drop. After the addition, the reaction mixture was left for 4 h at room temperature and then concentrated and Et_2O was added to precipitate the urea. The solid was filtrated, washed with a small amount of Et_2O, and dried under vacuum to give the urea **4j** (180 mg, 43%) as a white solid.

Synthesis of 1-(2-Aminophenyl)-3-(3-benzyloxypyridin-2-yl)-urea (5a). Compound **4b** (1 equiv, 0.30 mmol) was dissolved in absolute ethanol (0.008 M), and $SnCl_2$ dihydrate (6 equiv, 1.80 mmol) was added in one portion. The reaction mixture was heated under reflux for 4 h and the ethanol was evaporated. The residue was taken up with EtOAc, and the organic phase was washed with a solution of 1 M $NaHCO_3$ and brine, dried over Na_2SO_4, filtrated, and concentrated to afford amine **5a** as a white solid (90%). This product is unstable and it is used directly after isolation.

Synthesis of Amine 5b−c: General Procedure. Compounds **4c,d** (1 equiv) were dissolved in absolute ethanol (0.008 M), and $SnCl_2$ dihydrate (6 equiv) was added in one portion. The reaction mixture was heated under reflux for 4 h and the ethanol was evaporated. The residue was taken up with EtOAc, and the organic phase was washed with a solution of 1 M $NaHCO_3$ and brine, dried over Na_2SO_4, filtrated, and concentrated. The crude product was chromatographed on a silica gel column (ethyl acetate/cyclohexane/methanol 47.5/47.5/5). Evaporation and drying of the product afforded amine **5b,c** as a white solid.

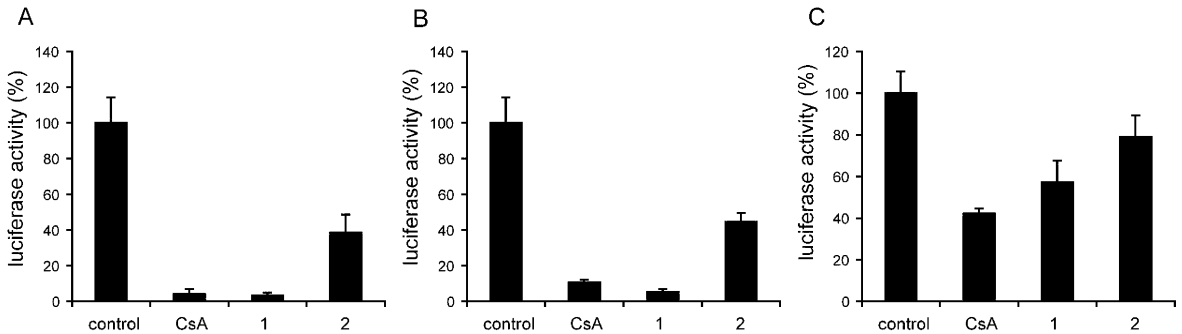


Figure 3. Effect of various compounds on HIV-1 infectivity. Viral infectivity was determined by one-round infection as described in the Experimental Section. Effects of CsA, **1**, and **2** were expressed as the percentage of luciferase activity of the control. Inhibitors were added in both producing and target cells (A), producing cells only (B) or target cells only (C). The results represent one of two independent experiments. Each experiment had been performed in triplicate.

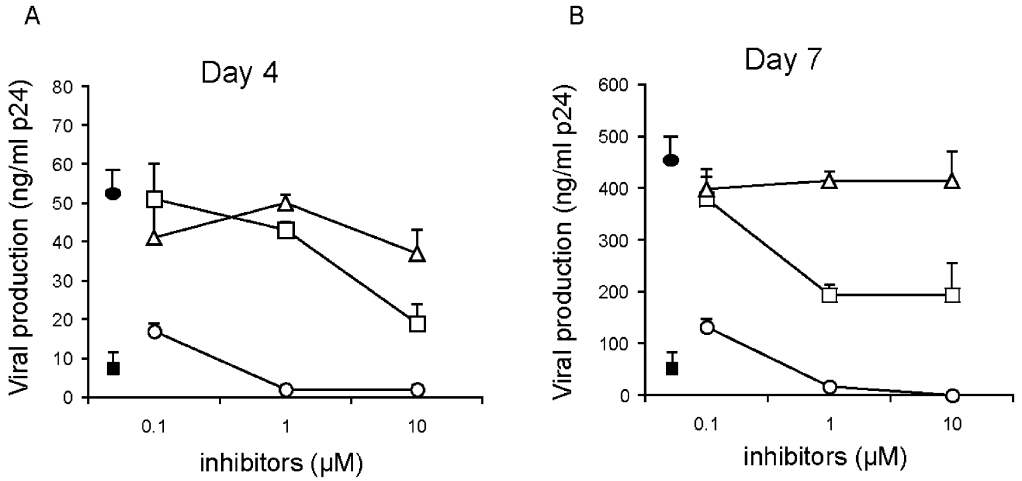


Figure 4. Effect of different compounds on HIV-1 production in human PBMC. Viral production is monitored by p24 ELISA at days 4 (A) and 7 (B) postinfection. Increasing concentration of CsA (○), **1** (□), or **2** (△) inhibitors were added to the culture medium. Viral productions of control (●) or 50 μM AZT (■) is shown with error bars.

Synthesis of Amide 6a−c: General Procedure. Compounds **5a−c** (1 equiv) were dissolved in THF (0.03 M), and Ac_2O (10 equiv) was added in one portion. The reaction mixture was left for 1 h at room temperature and the THF was evaporated. The crude product was chromatographed on a silica gel column (ethyl acetate/cyclohexane/methanol 45/45/10). Evaporation and drying of the product afforded amide **6a−c** as a white solid.

Synthesis of Urea 7a−c: General Procedure. Compounds **5a−c** (1 equiv, 0.12 mmol) were dissolved in THF (1 mL) and H_2O (2 mL), and then a solution of KOCN (2 equiv, 0.24 mmol) in 10% AcOH in H_2O (2 mL) is added. The reaction mixture was left for 2 h at room temperature and the solvent evaporated. The crude product was chromatographed on a silica gel column (ethyl acetate/cyclohexane/methanol 4/4/1). Evaporation and drying of the product afforded urea **7a−c** as a white solid.

Synthesis of Amide 8a−b: General Procedure. Compounds **4e,f** (0.15 mmol) was dissolved in THF/EtOH/DMF 1/1/1 (3 mL), and successively a solution of 3 N Na_2CO_3 (1 mL) and a 35% solution of H_2O_2 (0.9 mL) were added. After 4 days at room temperature, 4 mL of water was added and the reaction mixture was extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, filtrated, and concentrated. The crude product was triturated with Et_2O to precipitate the amide. The solid was filtrated, washed with a small amount of Et_2O, and dried under vacuum to give the amide **8a,b** as a white solid.

Synthesis of Benzoic Acid 9a−c: General Procedure. Compounds **4g−i** (1 equiv) were dissolved in THF/MeOH (1/1) (0.02 M) and a solution of 1 M NaOH (10 equiv) was added drop by drop. The reaction mixture was left overnight at room temperature. The reaction mixture was neutralized with a solution of 5 M HCl, and solvents were evaporated. The residue was taken up with 40 mL of water and this solution was saturated with NaCl. The aqueous phase was extracted with EtOAc (3 × 50 mL). The organic phases were combined, dried over Na_2SO_4, filtered, and concentrated to afford the benzoic acid **9a−c** as a white solid.

Synthesis of 3-[3-(3-Benzyloxypyridin-2-yl)ureido]benzoic Acid Methyl Ester (10a). Compound **4h** (1 equiv, 0.26 mmol) was dissolved in dry MeOH/THF (5/1) (0.02 M). A solution of 1.3 M sodium methanolate (10 equiv, 2.6 mmol) in dry methanol was added drop by drop. The reaction mixture was left for 1 h at room temperature and neutralized with a solution of 1 M HCl, and the solvents were evaporated. The residue was taken up with EtOAc, and the organic phase was washed with a solution of saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtrated, and concentrated to give the methyl ester **10a** as a white solid.

Synthesis of 4-[3-(3-Benzyloxypyridin-2-yl)ureido]benzenesulfonamide (11). Compound **4j** (41.7 mg, 0.1 mmol) was dissolved in 2 mL of dry THF and this solution was cooled with an ice bath. An aqueous solution of ammoniac (0.5 mL, 33%) was added drop by drop. After 15 min, the reaction mixture was concentrated and the residue was taken up with CH_2Cl_2. The organic phase was washed with brine, dried over Na_2SO_4, filtrated, and concentrated. The crude product was purified by flash chromatography (ethyl acetate/cyclohexane/methanol 7/2/1) to afford the sulfonamide **11** (6 mg, 17%) as a white solid.

Synthesis of 4-[3-(3-Benzyloxypyridin-2-yl)ureido]-*N*-methylbenzenesulfonamide (12). Compound **4j** (41.7 mg, 0.1 mmol) was dissolved in 2 mL of dry THF, and this solution was cooled with an ice bath. An aqueous solution of methylamine (0.5 mL, 40%) was added drop by drop. After 15 min, the reaction mixture was concentrated and the residue was taken up with CH_2Cl_2. The organic phase was washed with brine, dried over Na_2SO_4, filtrated, and concentrated. The crude product was purified by flash chromatography (ethyl acetate/cyclohexane/methanol 7/2/1) to afford the sulfonamide **12** (18 mg, 43%) as a white solid.

Synthesis of 5-[3-(3-Benzyloxypyridin-2-yl)ureido]isophthalamic Acid Methyl Ester (13) and 5-[3-(3-Benzyloxypyridin-2-yl)ureido]isophthalamide (14). Compound **4k** (100 mg, 0.23 mmol) was dissolved in 40 mL of MeOH. This solution was cooled with an ice bath and ammoniac was bubbled in for 15 min. The reaction mixture was left for 5 days at room temperature (each day ammoniac was bubbled in for 15 min with an ice bath). The reaction mixture was concentrated and the residue was taken up with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, filtrated, and concentrated. The crude products were purified by flash chromatography (ethyl acetate/cyclohexane/methanol 7/2/1) to afford **13** (4 mg, 4%) and **14** (20 mg, 20%).

Synthesis of 1-(3-Hydroxypyridin-2-yl)-3-phenylurea (15). Compound **4a** (1.0 g, 3.13 mmol) was dissolved in 70 mL of dry MeOH and 5 mL of dry THF. This solution was degassed with nitrogen and Pd/C (120 mg) was added. The resulting solution was submitted to a pressure of hydrogen (60 mbar) for 1 h. The reaction mixture was filtered (Celite) and the filtrate was concentrated. The residue was triturated with Et_2O, filtrated, and washed with a small amount of Et_2O to afford **15** as a white solid (458 mg, 64%).

Synthesis of Ether 16a,b: General Procedure. Compound **15** (115 mg, 0.5 mmol) was slowly added to a suspension of NaH in 4 mL of dry DMSO. After 30 min at room temperature, the reaction mixture was cooled with an ice bath and the alkylating agent (1.2 equiv, 0.6 mmol) was added drop by drop. The temperature of the resulting solution rose to room temperature. After 3 h, water was added and the reaction mixture was extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, filtrated, and concentrated. The crude product was purified by flash chromatography (ethyl acetate/cyclohexane 3/7) to afford the ether as a white solid.

Synthesis of 1-(3-Benzyloxypyridin-2-yl)-3-phenylthiourea (17). 2-Amino-3-benzyloxypyridine **3** (200 mg, 1.0 mmol) was dissolved in 4 mL of dry THF. The phenyl isothiocyanate (135 mg, 1,2 mmol) was added in one portion and the reaction mixture was heated under reflux for 72 h. The reaction mixture was concentrated and the crude product was purified by flash chromatography (ethyl acetate/cyclohexane 2/8) to afford the thiourea **17** (242 mg, 73%).

Synthesis of *N*-(3-Benzyloxypyridin-2-yl)benzamide (18). Benzoyl chloride (116 μL, 1 mmol) was dissolved in 10 mL of dry THF, and then a solution of 2-amino-3-benzyloxypyridine **3** (1.0 g, 5 mmol) in 10 mL of dry THF was added drop by drop. After the addition, the reaction mixture was left for 4 h at room temperature and then concentrated. The crude product was purified by flash chromatography (ethyl acetate/cyclohexane 4/6) to afford the amide **18** (241 mg, 81%) as a white solid.

Synthesis of *N*-(3-Benzyloxypyridin-2-yl)benzenesulfonamide (19). 2-Amino-3-benzyloxypyridine **3** (100 mg; 0.5 mmol) was dissolved in 10 mL of dry THF. Then benzene sulfonyl chloride (89 mg, 0.5 mmol) is added in one portion and the reaction mixture was heated under reflux for 40 h. The reaction mixture was concentrated, the residue was taken up with CH_2Cl_2, and the organic phase was washed with water and brine, dried over Na_2SO_4, filtrated, and concentrated. The crude product was purified by flash chromatography (EtOAc/cyclohexane 15/85) to afford the sulfonamide **19** (50 mg; 30%).

Ability of Compounds To Interfere with the HIV-1 Replication Cycle. Cells. HOS-CD4$^+$-CCR5$^+$ cells (AIDS Reagent Program) and SV40 T antigen-transformed human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics.

One-Round Infection Assay. To produce replication-defective HIV virions, 293T cells were cotransfected with the pNL4.3-env$^-$Luc$^+$ (AIDS Reagent Program) plasmid and with the pCMV-Ad8-Env plasmid encoding the envelope of HIV-1 prototype Ad8 with increasing amounts of CsA analogues. HOS-CD4$^+$-CCR5$^+$ cells were plated in a 24-well plate at a density of 5×10^4 cells/well. They were exposed to 30 ng of p24 equivalents of Ad8-pseudotyped defective virions for 18 h, washed, and cultured for 72 h. Luciferase activity was measured by luminometry, using the Luciferase Assay System (Promega).

Cell Infection. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and stimulated for 48 h with phytohemagglutinin (5 μg/mL, Difco Laboratories) and 100 U/mL of interleukin-2 (Boehringer-Mannheim). Cells (1.5×10^5) were then incubated for 1 h with or without the inhibitory compounds and exposed to 40 ng of p24 equivalent of the pNL4.3 HIV-1 strain for 24 h in 96-wells plates, washed extensively, and cultured at 2×10^6 cells/mL in 0.5 mL RPM-I1640 (GIBCO) supplemented with 10% fetal calf serum, antibiotics, glutamine, and with or without compounds. Half of the medium was changed with fresh medium every 3 days, and infection was monitored by p24 ELISA (Beckmann Coulter).

Inhibition of Isomerase Activity of Cyclophilin A. The cis−trans isomerization of an alanine-proline bond in a model substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, was monitored on a spectrophotometer (Varian) in a chymotrypsin-coupled assay. The inhibition of this reaction induced by different concentrations of inhibitor was determined, and the data were analyzed as a change in first-order rate constant as a function of inhibitor concentration, which yields the K_i value (GraphPad software). The following were added to a cuvette: 840 μL of ice cold assay buffer (40 mM HEPES, pH 7.9, 100 mM NaCl), 70 μL of Cyp A (0.36 μM in 40 mM HEPES, pH 7.9, 100 mM NaCl), 70 μL of chymotrypsin (50 mg/mL in 1 mM HCl), and 10 μL of test compound, at various concentrations, in dimethyl sulfoxide. The reaction was initiated by the addition of 10 μL of substrate (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, 2 mg/mL in 500 mM LiCl in trifluoroethanol). The absorbance at 390 nm versus time was monitored for 90 s using a spectrophotometer (Varian), and the rate constants were determined from the absorbance versus time data files.

Virtual Screening. The structure-based design of inhibitors was performed using the crystal structure of the CypA/CsA complex (pdb code 1CWA). A total of 3129 molecules with specific pharmacophoric constraints and molecular weights up to 650 Da was retrieved from the ACD (Available Chemicals Directory, Release ACD 2004−2, 296 387 compounds in total). To take into account the interatomic distances of the pharmacophore, the ACD-3D database that includes the 3D atomic coordinates was used. This set of molecules retrieved in a multi-SDF file were converted to 3D structures with CORINA (Molecular Networks GmbH) and then docked and evaluated by the FlexX program.[23] Standard parameters of the FlexX 1.13.1 program were used for iterative growing and subsequent scoring of FlexX poses. Active-site atoms were defined as a sphere of 10 Å from the center point defined by Phe113. A receptor description file was automatically defined from the pdb coordinates of the hydrogen-free protein/active site coordinates. Molecules with a score higher than −25 and docked further than 10 Å of the Phe113 side chain of CypA were eliminated by using the LEAD3D program developed by Douguet et al.[37] All poses were rescored with X-Score[23] to rerank them and to give a more accurate estimation of the binding free energies of the molecules. Inspection of the hit lists and their docked structures fitted into the complex were visualized with the program XmMol.[38] Visual inspection of the suggested binding modes of FlexX, according to the scoring values of FlexX and X-Score, was used to select a set of compounds to be ordered and tested. In a second set of experiments, each molecule found to be active against the isomerase activity of cyclophilin A was docked and evaluated with another docking program called Surflex.[24] This new docking study between the CypA structure and, for example, compounds **1** or **2** was realized to define more accurately the putative binding mode of the compounds. It has to be noticed that for virtual screening calculations performed with FlexX and Surflex, no water molecules were added during the binding-site or the protomol generation, respectively.

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Supporting Information Available: Table providing results from purity analyses by HPLC for key target compounds and chemical shifts of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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