Bioorganic & Medicinal Chemistry Letters 22 (2012) 2308–2311

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Substituent effects on P2-cyclopentyltetrahydrofuranyl urethanes: Design, synthesis, and X-ray studies of potent HIV-1 protease inhibitors

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ARTICLE INFO

Article history:
Received 28 December 2011
Accepted 17 January 2012
Available online 2 February 2012

Keywords:
HIV-1 protease inhibitors
P2 ligand
Drug resistance
Design and synthesis
X-ray crystal structure

ABSTRACT

The design, synthesis, and biological evaluation of novel C3-substituted cyclopentyltetrahydrofuranyl (Cp-THF)-derived HIV-1 protease inhibitors are described. Various C3-functional groups on the Cp-THF ligand were investigated in order to maximize the ligand-binding site interactions in the flap region of the protease. Inhibitors **3c** and **3d** have displayed the most potent enzyme inhibitory and antiviral activity. Both inhibitors have maintained impressive activity against a panel of multidrug resistant HIV-1 variants. A high-resolution X-ray crystal structure of **3c**-bound HIV-1 protease revealed a number of important molecular insights into the ligand-binding site interactions.

HIV-1 protease inhibitors continue to be a critical component of frontline therapy in the treatment of HIV patients.[1–3] Our continuing studies on the structure-based design of inhibitors targeting the protein backbone led to the discovery of a variety of novel HIV-1 protease inhibitors (PIs) with broad-spectrum activity against multidrug-resistant HIV-1 variants.[4–8] We recently reported various C3-functionalized cyclopentyltetrahydrofuran (Cp-THF)-derived P2-ligands designed to specifically interact with the flap Gly48 amide NH in the S2-subsite of the HIV-1 protease.[9] One of these inhibitors, **2** (Fig. 1), containing a 3-(*R*)-hydroxy group on the Cp-THF core displayed exceptionally potent enzyme inhibitory (K_i = 5 pM) and antiviral activity (IC_{50} = 2.9 nM). This inhibitor also exhibited potent activity against a panel of multidrug-resistant HIV-1 variants. The X-ray crystal structure of **2**-bound HIV protease revealed an extensive hydrogen-bonding network with the enzyme backbone.[9] Of particular interest, the 3-(*R*)-hydroxy group of the Cp-THF ligand was involved in an interesting water-mediated interaction with the backbone NH amide bond of Gly48. This specific interaction was not present in inhibitor **1**. These additional interactions observed with **2** may have contributed toward its impressive drug resistance profile. [9]

Based upon the **2**-bound X-ray crystal structure of HIV-1 protease, and given the significant gain in antiviral activity observed with the addition of C3-polar substituents on the Cp-THF P2 ligand, we subsequently speculated that N-substituted functionalities, particularly *N*-acyl, *N*-carbamate or *N*-sulfonyl derivatives could function as both a hydrogen-bonding donor and acceptor. The NH proton could conceivably form an effective hydrogen bond with the proximal Gly48 carbonyl while amide or urethane carbonyl oxygen may form an additional interaction with the protease backbone. Indeed, our previous exploration of such hydrogen bond donor and acceptor functionalities on P2-ligand frameworks led to remarkably potent HIV-1 protease inhibitors with broad-spectrum antiviral activity. [4–9] Herein, we report the design, synthesis and biological evaluation of a series of HIV-1 protease inhibitors with C-3 substituted Cp-THF as the P2-ligand. A number of inhibitors exhibited exceptionally potent antiviral activity against a panel of multidrug-resistant HIV-1 variants. A protein-ligand X-ray crystal structure also provided important molecular insight into the ligand-binding site interactions.

The synthesis of ligands containing various N-substituents with either stereochemistry at C3 was accomplished starting from our previously reported optically active ketone intermediate **4**[10] as shown in Scheme 1. Ketone **4** was converted to methyloxime derivatives **5** in 96% yield. Reduction of **5** with a mixture of Pd/C and Raney-Ni under hydrogen pressure (80 psi) provided the corresponding amine as a 3:1 diastereomeric mixture.[11] The amine mixture was reacted with Ac_2O in the presence of Et_3N and a catalytic amount of DMAP to yield a mixture of isomeric TBS-protected

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doi:10.1016/j.bmcl.2012.01.061

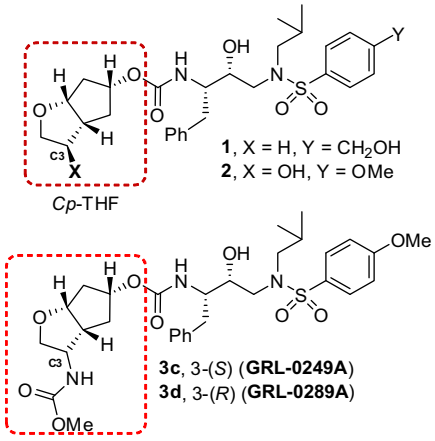
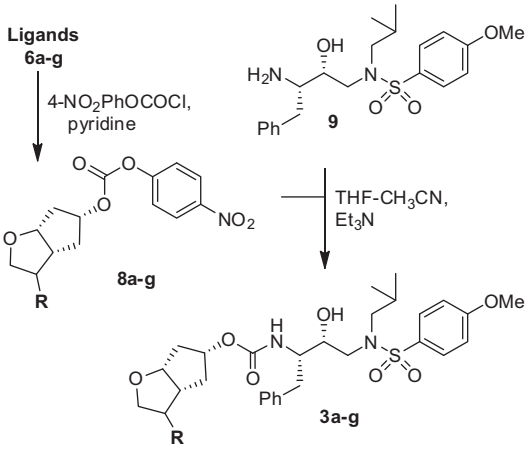
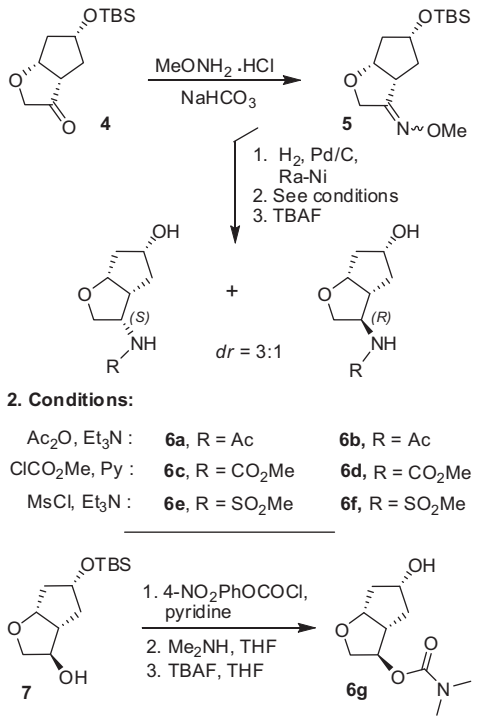


Figure 1. Structure of protease inhibitors **1,2**, **3c–d**.



Scheme 2. Synthesis of protease inhibitors **3a–g**.



Scheme 1. Synthesis of C3-substituted ligands **6a–g**.

amide intermediates in 80% yield. Treatment of the respective amides with TBAF in THF and chromatographic separation furnished diastereomerically pure ligands **6a** and **6b** in excellent yield. Similarly, the amine mixture was treated with methyl chloroformate and pyridine, or mesyl chloride and Et_3N to afford the corresponding carbamates and sulfonamides. Treatment of the respective crude mixtures with TBAF in THF followed by chromatographic separation afforded diastereoisomeric *N*-carbamate ligands **6c** and **6d**, and *N*-mesyl ligands **6e** and **6f**, respectively. The assignment of stereochemistry on the ligands was carried out by NOE or NOESY experiments of the corresponding mixed activated carbonates **8a**, **8c**, and **8e**. To probe the importance of the free NH, we have synthesized ligand **6g** containing a 3-(*R*)-*O*-dimethylaminocarbamate group. This was synthesized in three consecutive steps starting from our previously reported optically active alcohol **7**.[9] Treatment of **7** with 4-nitrophenyl chloroformate in the presence of pyridine furnished the corresponding mixed activated carbonate. The resulting carbonate was reacted with a bubbling stream of Me_2NH gas to provide the corresponding TBS-protected ligand. Removal of the TBS-group with TBAF furnished ligand **6g** in excellent yield.

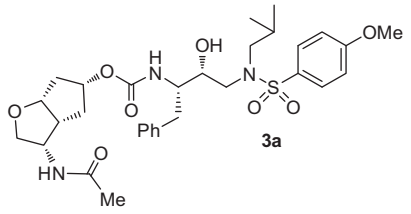
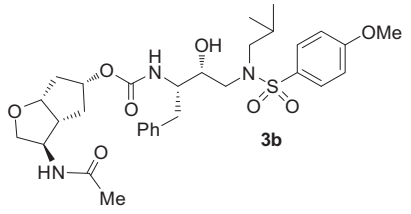
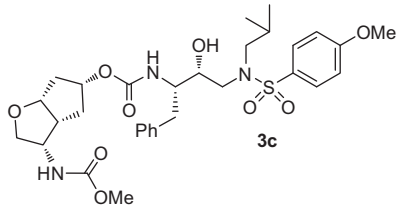
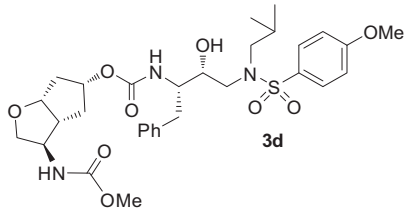
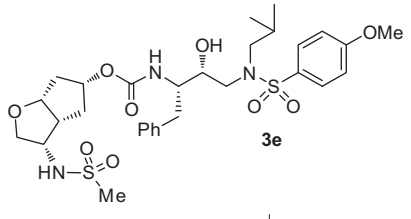
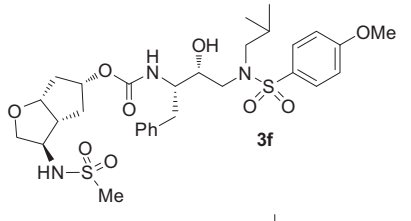
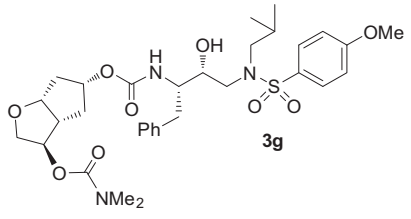
For the synthesis of HIV protease inhibitors, all ligand alcohols **6a–g** were reacted with 4-nitrophenyl chloroformate in the presence of pyridine in CH_2Cl_2 to furnish the corresponding mixed activated carbonates **8a–g** (Scheme 2).[9,10] The activated carbonates were then reacted with previously reported hydroxyethylamine isostere **9**[9] in the presence of Et_3N in THF/CH_3CN for 2–4 days to give corresponding inhibitors **3a–g**.

Inhibitors **3a–g** were initially tested in enzyme inhibitory assays using the method developed by Toth and Marshall,[12] and then evaluated for in vitro antiviral assays. Results are shown in Table 1. All inhibitors displayed impressive inhibitory potency and high antiviral activity. Inhibitors **3a** and **3b** that, contain a 3-(*S*)- or 3-(*R*)-*N*-acetyl substituent on the Cp-THF ligand, exhibited similar potency (7.4 and 7.5 pM, respectively). Interestingly, the stereochemistry at C3 seemed to have little effect. Inhibitor **3c** with a 3-(*S*)-*N*-methoxycarbonyl displayed the most impressive enzyme and antiviral potency (K_i = 1.8 pM and IC_{50} = 1.6 nM). The isomeric inhibitor **3d** provided slightly lower potency. Inhibitors **3e** and **3f** that contain a 3-*N*-mesyl on the Cp-THF ligand, showed a substantial reduction in activity probably due to the increase in steric bulk created by the *N*-mesyl group. Inhibitor **3g** that contains an O-substituted dimethylaminocarbamate in place of N-substituted carbamate at C3 provided a good antiviral activity, similar to that of **3d**.

Inhibitors **3c** and **3d**, were further evaluated against a panel of multidrug-resistant (MDR) HIV-1 variants and their antiviral activities were compared to clinically available PI, darunavir (DRV).[6,13] Results are shown in Table 2. All inhibitors exhibited low nanomolar EC_{50} values against the wild-type HIV-$1_{ERS104pre}$ laboratory strain, isolated from a drug-naïve patient.[13] Inhibitor **3d** had the most potent activity (EC_{50} = 3 nM) similar to that of DRV. When tested against a panel of multidrug-resistant HIV-1 strains, the EC_{50} of **3d** remained in the low nanomolar value range (15–24 nM) and its fold-changes in activity were similar to those observed with DRV.[6,13] Interestingly, inhibitor **3c**, with the opposite (*S*) stereochemistry at C3, displayed slightly lower antiviral activities against all viral strains compared to **3d**. However, the fold-changes in EC_{50} for **3c** remained low (<3) against all MDR HIV-1 viruses. The fold-changes contrasted with those of **3d** and even DRV, for which the respective EC_{50}'s increased by a factor of at least three against the MDR viruses examined.[14]

In order to gain molecular insights on the ligand-binding site interactions responsible for the potent activity and excellent resistance profile of **3c**, we have determined the X-ray crystal structure

Table 1
Structures and potency of inhibitors **3a**–**g**[a]

Entry	Inhibitor structure	K_i (pM)	IC_{50} (nM)[b]
1	 **3a**	7.4	25
2	 **3b**	7.5	31
3	 **3c**	1.8	1.6
4	 **3d**	4.0	4.6
5	 **3e**	32	28
6	 **3f**	180	50
7	 **3g**	20	4.7

[a] Values are the mean value of at least two experiments.
[b] Human T-lymphoid (MT-2) cells (2×10^3) were exposed to 100 $TCID_{50}$ of HIV-1_{LAI} and cultured in the presence of each PI, and IC_{50} values were determined using the MTT assay. The IC_{50} values of amprenavir (APV), saquinavir (SQV), indinavir (IDV), and darunavir (DRV) were 0.03, 0.015, 0.03, and 0.003 μM, respectively.

of the HIV wild-type protease complexed with **3c** (Fig. 2).[15] The structure was refined to an R-factor of 14.9% and a resolution of 1.23 Å. The inhibitor is bound to the protease dimer in two orientations related by a 180° rotation, with a 50/50 relative occupancy. The protease backbone structure showed a very low rms deviation of 0.11 Å for all Cα carbons compared to the protease complexes of **2**[9] or DRV.[21] As shown in Figure 2, the inhibitor interactions in the protease binding site extend from S2 to S2′ protease subsites and consist of a series of strong hydrogen bonds and weaker C–H···O and C–H···π interactions similar to those previously described for DRV,[21] or inhibitor **2**.[9] The Cp-THF cyclic oxygen forms a strong hydrogen bond with the backbone amide NH of Asp29 in the protease S2-binding site, similar to that previously observed with other Cp-THF-based inhibitors **1**[5] and **2**.[9] Critical differences, however, are observed with the additional interactions that the 3-(*S*)-*N*-methoxycarbonyl amino substituent on the Cp-THF makes throughout the S2–S3 subsites. As shown in Figure 2, the carbamate NH forms a strong hydrogen bond with the Gly48 backbone carbonyl. The carbamate carbonyl is observed to interact with the Arg8′ guanidine side chain through a conserved water molecule. The methyl of the methoxy group then fits within the S3-hydrophobic pocket. The C3-*N*-methoxycarbonyl amino group creates a network of tight hydrogen bonds that literally links the protease flap region to the S2–S3 subsites' dimer interface. These new interactions and enthalpic nature of the additional hydrogen bonding created by the P2-ligand may certainly exert an enhanced anchoring effect of the inhibitor into the S2-subsite and further stabilize the closed conformation of the protease–ligand complex.

Table 2
Comparison of the antiviral activity of **3c**, **3d**, and DRV against multidrug-resistant HIV-1 variants

Virus[a]	EC_{50} (μM) ±SDs, (fold-change)[b]		
	3c	**3d**	**DRV**
HIV-$1_{ERS104pre}$ (wt)	0.029 ± 0.002	0.003 ± 0.001	0.004 ± 0.001
HIV-$1_{MDR/B}$ (X4)	0.075 ± 0.011 (3)	0.018 ± 0.003 (6)	0.019 ± 0.006 (5)
HIV-$1_{MDR/C}$ (X4)	0.030 ± 0.006 (1)	0.015 ± 0.005 (5)	0.011 ± 0.003 (3)
HIV-$1_{MDR/G}$ (X4)	0.039 ± 0.001 (1)	0.020 ± 0.005 (7)	0.011 ± 0.002 (3)
HIV-$1_{MDR/TM}$ (X4)	0.074 ± 0.006 (3)	0.024 ± 0.004 (8)	0.028 ± 0.001 (7)

[a] Amino acid substitutions identified in the protease-encoding region of HIV-$1_{ERS104pre}$, HIV-$1_{MDR/B}$, HIV-$1_{MDR/C}$, HIV-$1_{MDR/G}$, HIV-$1_{MDR/TM}$ compared to the consensus type B sequence cited from the Los Alamos database include L63P in HIV-$1_{ERS104pre}$; L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, I93L in HIV-$1_{MDR/B}$; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89M in HIV-$1_{MDR/C}$; L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90M in HIV-$1_{MDR/G}$; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, I93L in HIV-$1_{MDR/TM}$. HIV-$1_{ERS104pre}$ served as a source of wild-type HIV-1.
[b] EC_{50} values were determined by using PHA-PBMs as target cells and the inhibition of p24 Gag protein production for each drug was used as an endpoint. The numbers in parentheses represent the fold-change in EC_{50} values for each isolate compared to the EC_{50} values for the wild-type HIV-$1_{ERS104pre}$. All assays were conducted in duplicate, and the data shown represent mean values (±1 standard deviations) derived from the results of two or three independent experiments. PHA-PBMs were derived from a single donor in each independent experiment. DRV (darunavir).

In conclusion, we have reported the structure-based design of a series of highly potent HIV-1 protease inhibitors incorporating C3-substituted cyclopentyltetrahydrofuranyl urethanes as P2-ligands. Various C3-*N*-substituents were investigated in order to create multiple interactions in the S2-subsite of the protease and specifically with the protease flap region. Inhibitors **3c** and **3d** displayed remarkable inhibitory potency and antiviral activity. When tested against a panel of MDR HIV-1 strains, inhibitor **3d**, with a 3-(*R*)-methoxycarbonyl on the Cp-THF ligand, provided the most impressive EC_{50}s and fold-changes in activity which are comparable to those observed with clinically available DRV. Isomeric inhibitor **3c** displayed lower antiviral activity. However, it exhibited strikingly low fold-changes of antiviral activity when tested against MDR HIV-1 viruses. An X-ray crystal structure of the protease–**3c**

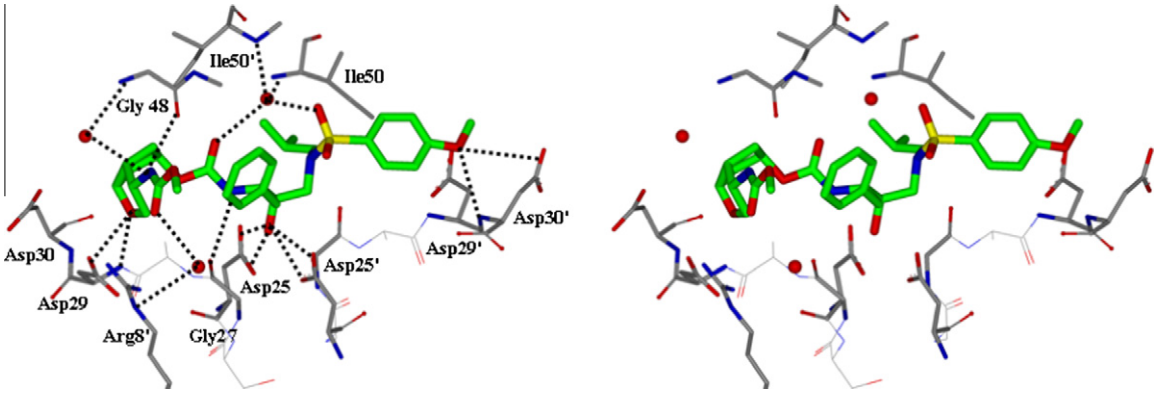


Figure 2. Stereoview of the X-ray structure of inhibitor **3c** (green)-bound HIV-1 protease (PDB code: 4DFG). All strong hydrogen bonding interactions are shown as dotted lines.

complex was determined at a 1.23 Å resolution. Inhibitor **3c** made extensive interactions throughout the protease binding site. The complex network of hydrogen-bonding interactions created by the *N*-methyl carbamate substituent in addition to those created by the isostere in the protease active site may account for the impressive antiviral activity and superb resistance profile observed with inhibitor **3c**. Further designs along this line and ligand optimization are currently underway.

Acknowledgements

This research was supported by the National Institutes of Health (Grant GM53386 to A.K.G. and Grant GM62920 to I.T.W.). This work was also supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health, and in part by a Grant-in-Aid for Scientific Research (Priority Areas) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Monbu Kagakusho), a Grant for Promotion of AIDS Research from the Ministry of Health, Welfare, and Labor of Japan, and the Grant to the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Reemerging Infectious Diseases (Renkei Jigyo) of Monbu-Kagakusho.

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15. The protein-ligand X-ray structure of **3c**-bound HIV-1 protease will be deposited in PDB (PDB ID: 4DFG). The HIV-1 protease was expressed and purified as previously described.[16] The protease–inhibitor complex was crystallized at room temperature by the hanging drop vapor diffusion method with well solutions of 1.2 M ammonium chloride and 0.1 M sodium acetate buffer (pH 4.8). Diffraction data were collected on a single crystal cooled to 90 K at SER-CAT BM beamline 22, Advanced Photon Source, Argonne National Laboratory (Chicago, IL, U.S.), with an X-ray wavelength of 1.0 Å and processed by HKL-2000 with R_{merge} of 7.2%. The PR structure was used in molecular replacement by PHASER[17] in the CCP4i suite[18] and refined to 1.45 Å resolution using SHELX-97 and COOT[19] for manual modification. PRODRG-2[20] was used to construct the inhibitor and the restraints for refinement. Alternative conformations were modeled, anisotropic atomic displacement parameters (*B* factors) were applied for all atoms including solvent molecules, and hydrogen atoms were added in the final round of refinement. The final refined solvent structure comprised two Cl^- ions and 142 water molecules.
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