New heteroaryl carbamates: synthesis and biological screening *in vitro* and in mammalian cells of wild-type and mutant HIV-protease inhibitors.

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In honor of professor Carlo Bonini for his 70° birthday

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Abstract:

New heteroaryl HIV-protease inhibitors bearing a carbamoyl spacer were synthesized in few steps and high yield, from commercially available homochiral epoxides. Different substitution patterns were introduced onto a given isopropanoyl-sulfonamide *core* that can have either H or benzyl group. The *in vitro* inhibition activity against recombinant protease showed a general beneficial effect of both carbamoyl moiety and the benzyl group, ranging the IC_{50} values between 11 and 0.6 nM. In particular, benzofuryl and indolyl derivatives showed IC_{50} values among the best for such structurally simple inhibitors. Docking analysis allowed to identify the favorable situation of such derivatives in terms of number of interactions in the active site, supporting the experimental results. The inhibition activity was also confirmed in HEK293 mammalian cells and was maintained against protease mutants. Furthermore, the metabolic stability was comparable with that of the commercially available inhibitors.

1. Introduction

The AIDS epidemic is still one of the most challenging problems although great efforts are made to the discovery of new drugs for its treatment.[1] Among many strategies to combat the disease, highly active antiretroviral therapy (HAART) containing at least one of HIV-1 protease inhibitors (PIs) is considered as the most effective treatment.[2]

In fact, the HIV-1 aspartic protease (HIV-PR) is essential for the production of mature HIV particles and plays a key role in maintaining infectivity. Currently, nine approved PIs are

commercially available and have definitely improved both the quality of life and life expectancy of HIV-infected patients. However, because of the rapid genomic evolution of the HIV, an inevitable consequence in the treatment of the infection has been the rise of drug resistance and therefore the dramatically reduction of the commercial inhibitors efficiency.

Thus, both the emergence of highly mutated viral strains cross-resistant to antivirals and the occurrence of various debilitating side effects, and not last the high cost of HAART, prompted scientists to seek novel PIs, desirably with alternative frameworks.

The last commercially available inhibitor, Darunavir, is actually the only one active against mutated virus; moreover, it is particularly interesting because of its double inhibitory activity: inhibition of the dimerization and inhibition of proteolytic activity.[3]

During our investigation on new peptidomimetics and non peptidic inhibitors, we found beneficial effect of heteroaryl rings as P2 ligand.[4]

In particular, the systematic study on derivatives of simple substituted stereodefined isopropanolamine core showed the high effect of the functionality between the heteroaryl group and the *core* together with the type of heteroaryl group. Promising IC_{50} value was obtained with benzothienyl derivative **1** (Figure 1).[4c] With the aim to take advantage of an enhancement of H-bond interactions, we synthesized in few steps and with high yield new simple benzofuryl, indolyl and benzothienyl HIV protease inhibitors **2** bearing a carboxyamide spacer, which in general showed *in vitro* activity against native protease, with IC_{50} values in the range of 1–15 nM. Such derivatives also showed inhibition activity in mammalian cells, demonstrating their promising potential.[5]

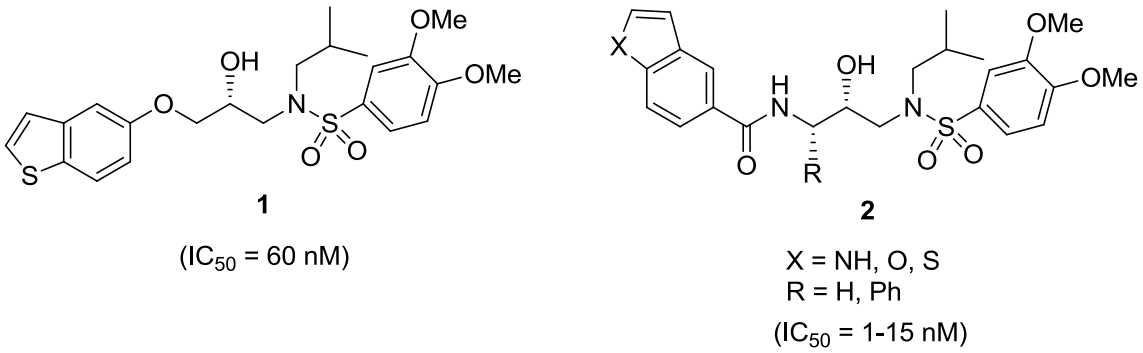


Figure 1

In our recent studies,[4a] some carbamoyl structures containing indole ring as heteroaryl mojety (compound **3**, figure 2) were examined, but the biological activity resulted only in the μM range.

As it can be seen, compound **3** bears the carbamoyl group, which is reversed respect to the well-known Darunavir and TMC-126

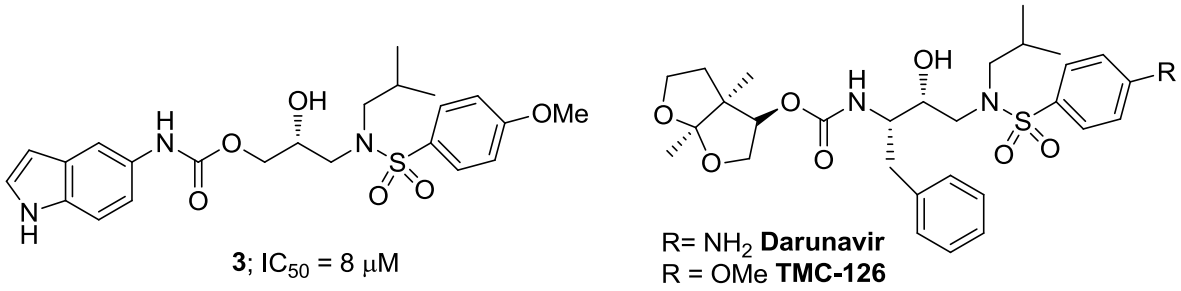
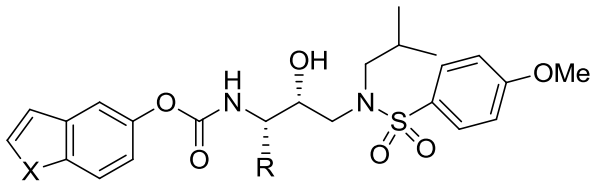


Figure 2

Thus, following the concept of targeting the protein backbone, with the aim to design simple non-peptidic heteroaryl structures, we planned a systematic study on the synthesis and inhibition activity of new derivatives with general structure **A**, in which the heterocycle is spaced from the core by a carbamoyl function in the same disposition of Darunavir and TMC-126. The type of heteroaryl group (X = O, NH, S) and the core were modified, with the presence of either H or benzyl as R substituent.



A

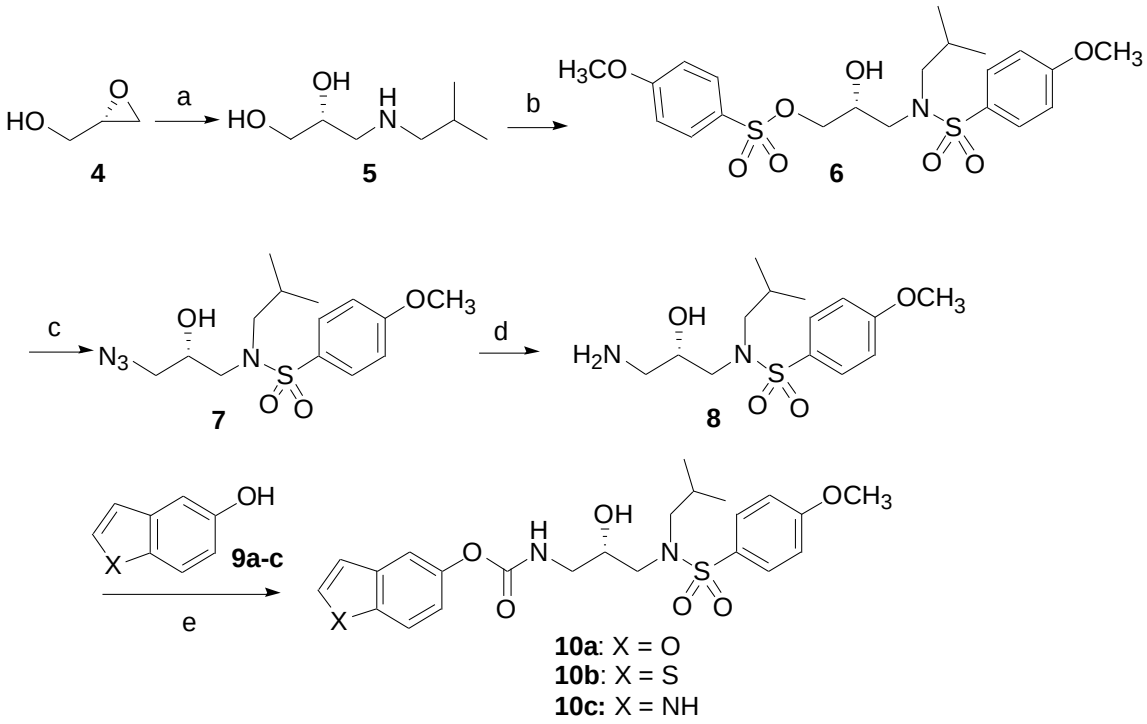
X = NH, O, S
R = H, Ph

Figure 3

2. Results and Discussion

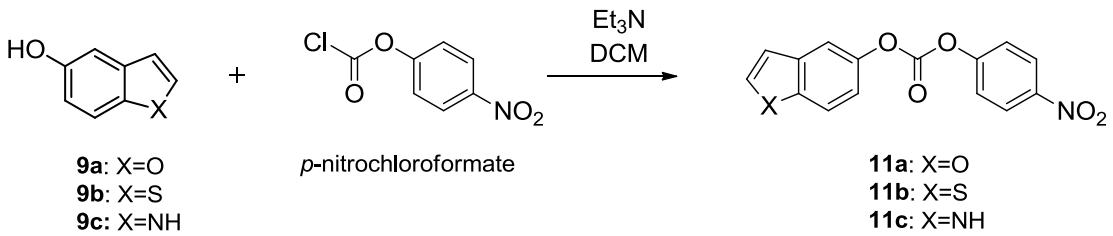
Chemistry

For the preparation of simple unsubstituted isopropanolamine core (R = H in figure 3), in consideration of our previous research,[4] the synthetic route started from the commercially available bidentate electrophile (*S*)-glycidol **4** (Scheme 1).



Scheme 1. Synthesis of inhibitors **10a-c:** (a) *i*-$BuNH_2$, *i*-PrOH, rt, 24h (quant.); (b) 2 eq. 4-methoxybenzenesulfonyl chloride, Et_3N, CH_2Cl_2, rt, 24h; (c) NaN_3, DMF, reflux (80% yield from **4**); (d) H_2, Pd/C 10%, EtOH, rt, 4h; (e) 5-hydroxyheteroarenes **9a-c**, *p*-nitrophenylchloroformiate, Et_3N, DCM, 1h, rt; then **8**, rt, 24h (**10a**, 74%, **10b**, 72%, **10c** 72%).

(*S*)-Glycidol was first reacted with isobutylamine in isopropanol to achieve aminodiol **5**, which was successfully treated with *p*-methoxybenzenesulfonyl chloride and triethylamine in dry CH_2Cl_2 to obtain sulfamido-sulfonate **6**. This compound underwent to nucleophilic displacement with NaN_3, to give the sulfamidyl-azide **7**, which was then reduced to amine **8** by catalytic hydrogenation. In the last step, the suitable heteroaryl-carbamoyl group was introduced by a reaction with different carbonates **11a-c**, which were formed *in situ* from the corresponding 5-hydroxyheteroarene **9a-c** and *p*-nitrophenylchloroformate. (Scheme 2)

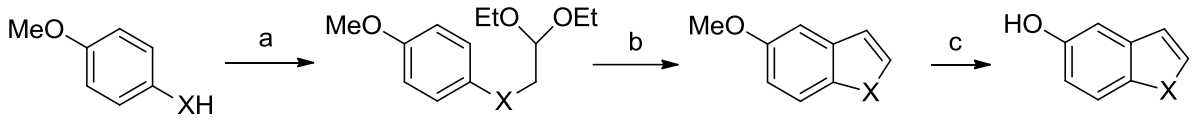


Scheme 2. Synthesis of intermediates **11a-c**

While 5-hydroxyindole **9c** is cheaply available, 5-hydroxybenzofuran **9a** and 5-hydroxybenzothiophene **9b** had to be prepared, due to their high cost. Unfortunately, only few

methods are reported in literature.[6] In particular, those starting from 4-methoxyphenol **12a** or 4-methoxythiophenol **12b** usually give low overall yield, due to the cyclization step. Hence, we successfully revisited such procedure, using bromodiethylacetal in the alkylation step and Amberlyst-15[7] in the subsequent cyclization/aromatization step. (Scheme 3).

By these modifications 5-methoxy-heteroarenes **14a-b** were obtained in good yield (> 60% in two steps from **12a-b**). Demethylation of **14a-b** afforded to desired 5-hydroxyheteroarenes **9a-b** in high yield, making such three steps procedure the best one reported, in terms of overall yield (> 49% yield from **12a-b**).



12a: X=O
12b: X=S

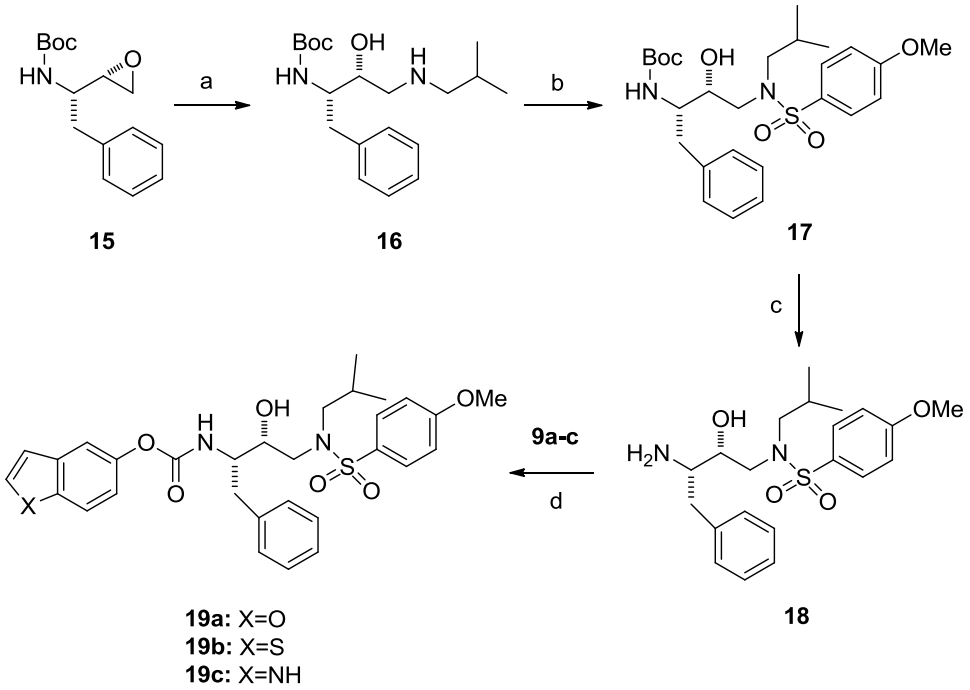
13a: X=O
13b: X=S

14a: X=O
14b: X=S

9a: X=O
9b: X=S

Scheme 3. Synthesis of heteroarenes **9a-b:** (a) 4-methoxy(thio)phenol **12a** or **12b**, $BrCH_2CH(OEt)_2$, Cs_2CO_3, MeCN, reflux, 40h (**13a**, 78%; **13b**, 81%); (b) **12a-c**, Amberlyst-15, chlorobenzene 120°C, 3h (**14a**, 78%; **14b**, 75%); (c) BBr_3SMe_2 1M in CH_2Cl_2, chlorobenzene 120°C, 24h (**9a**, 80%; **9b**, 81%).

The preparation of benzyl substituted derivatives (with R = Bn in structure A, figure 3) was even shorter, taking advantage of established chemistry[8] and using homochiral *N*-Boc protected amino epoxide **15**. The epoxide was firstly opened with *iso*-butylamine to afford the monoprotected diaminoalcohol **16** (scheme 4). Then, the *p*-methoxyphenylsulfonyl group was introduced and the *N*-Boc group efficiently displaced by TFA. The crude ammonium trifluoroacetate derivative was treated with NEt_3, affording the free amine **18**. From this common intermediate, we were easily able to achieve new inhibitors **19a-c** by reaction of amine **18** with the *in situ* preformed carbonates **11a-c** of the starting phenols **9a-c.**



Scheme 4. Synthesis of inhibitors **19a-c**: (a) *i*-BuNH_2, *i*-PrOH, 60°C, 4h (>99% yield); (b) 4-methoxybenzenesulfonyl chloride, Et_3N, CH_2Cl_2, rt, 24h; (c) TFA/CH_2Cl_2 30%, rt, 1 h; then Et_3N (84% yield from **16**); (d) 5-hydroxyheteroarenes **9a-c**, *p*-nitrophenylchloroformiate, Et_3N, CH_2Cl_2, 1h, rt; then **18**, rt, 24h (**19a**, 75%; **19b**, 71%; **19c** 70%).

It is to be highlighted that this synthetic pathway appears very solid, high yielding and general, irrespective on the *N*-alkyl group, the sulfonamide or the type of heteroaryl moiety chosen. The easy access of common intermediates represents an open door to different target molecules with pharmacological activities even beyond the inhibition of HIV-protease.[9]

Biological assays were carried out on all the prepared compounds both *in vitro* and in mammalian cells, as described in the next section.

In vitro activity

IC_{50} values were obtained on recombinant wild type HIV protease (Table 1) by measuring the initial rates of hydrolysis of the fluorogenic substrate Abz-Thr-Ile-Nle-Phe(NO2)-Gln-Arg as previously reported[5].

Table 1. Biological data (IC_{50}, CC_{50} and Clearance) of the synthetized inhibitors

Entry	Structure	IC_{50} (nM)	CC_{50} (μM)	Clearance (μL/min/mg protein): a) human liver microsome b) rat liver microsomes
1	**10a**	15400 ± 500		/
2	**10b**	49±7	>100	/
3	**10c**	13400 ± 220		/
4	**19a**	≤ 0.6*	>100	a) 121 b) 26.8
5	**19b**	10.8 ± 1.2	n.d.	n.d.
6	**19c**	≤ 0.6*	2.8	a) 548 b) 522

(*) as these values are very close to one half of the enzyme concentration used in the assay, they should be regarded as estimated upper limits.

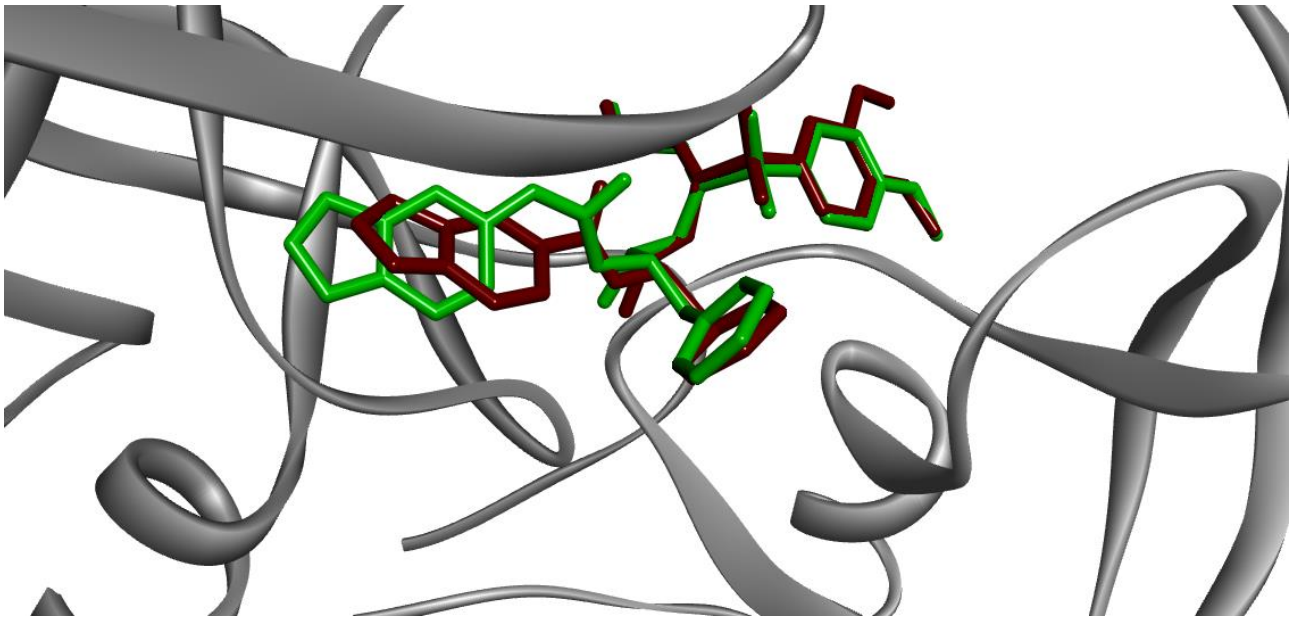
As it can be seen, compounds **19a** and **19c** are the most powerful inhibitors on the list, their IC_{50} values being as less that 0.6 nM. Their activity is therefore higher than that of their corresponding amides, described in our previous work.[5]

On the other hand, when the benzyl side chain at P1' is lacking, as in compounds **10a-c**, the activity is largely lost, with the exception of benzothienyl derivative **10b**. Under this point of view, this series of carbamates behaves differently from the previous series of amides, where also the benzofurane derivative corresponding to **10a** was found to inhibit the enzyme with an IC_{50} value of 1 nM.

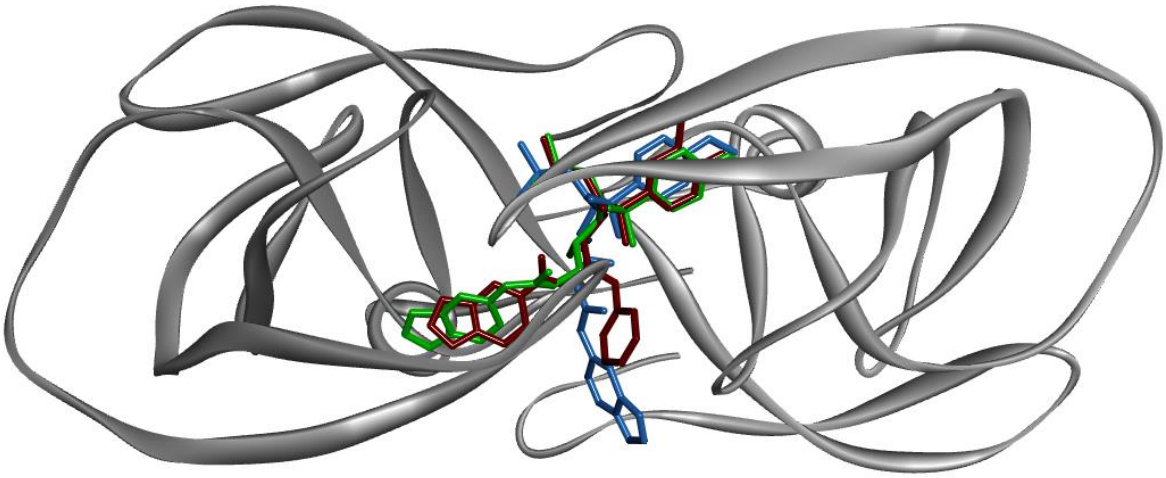
In order to explain such results, we carried out a series of docking runs on carbamates **19a**, **19c** and **10a**, **10c**. The calculation was carried out with Autdock Vina.[10] The best poses were further refined by MD runs, which were carried out with the Gromacs package.[11]

Despite the longer linker connecting the heteroaryl moiety with the core unit of the inhibitors, both **19a** and **19c** can interact very well with the S2' subsite, and the number of favorable interactions is indeed the same obtained with the corresponding amides already described,**Errore. L'origine riferimento non è stata trovata.** as there is room enough in the site. Moreover, the benzofuran system of **19a** could accept hydrogen bonding from Asp130 either at its side chain or at its backbone NH (Figure 4A, table 2). The lack of a methoxy group in comparison to the amides at the other side of the molecule has no effects on binding, most likely because this group was partly exposed to the solvent and not involved in interactions in the previous series of compounds. On the contrary, when the benzyl

group is removed from the molecules as in **10a** and **10c**, two main docking solutions are found, but both fail to reach the maximum number of interactions attainable with the amides or with reference inhibitors as Amprenavir. In the first option, the heterocyclic side chain is still found inside S2', with similar interactions, but of course the S1' subsite is completely empty. The second solution is similar to that obtained in our previous work[5] on the compounds lacking of the benzyl group, but this time the connecting chain is too long, and the aromatic system is largely exposed to the solvent outside the catalytic site of the enzyme, without recovering favorable interactions, as it can be clearly seen in the top view of Figure 4B and in table 2.



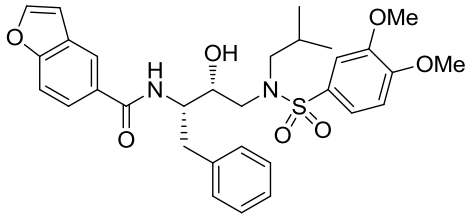
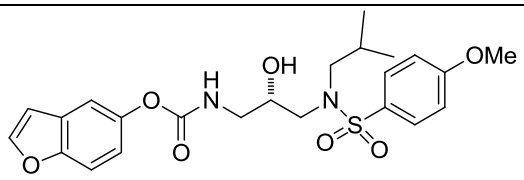
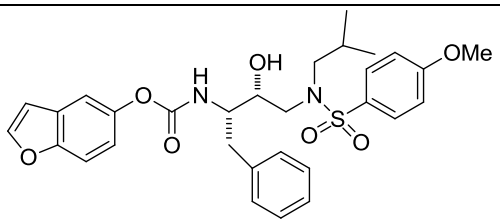
A



B

Figure 4. A: overlay of the optimized complex of HIV-protease with compound **19a** (green) with that of corresponding benzofuryl amide (red). **B**: top view of the overlay of the best docking solutions for compound **10a** (green and blue) with the corresponding amide (red).

Table 2. Number of favorable contacts established by the inhibitors at the S1' and S2' of the protease.

Inhibitor	S1'	S2'
Amprenavir	4	11
 **2**	2	9
 **10a**	0	8
 **19a**	2	9

Provided the presence of a new carbamoyl moiety, which makes these new compounds completely non peptidic, a preliminary study on the clearance was realized, in particular on inhibitors **19a** and **19c**. It is well known that all HIV-1 Pr inhibitors which are currently on the market, except Ritonavir, undergo rapid degradation by cytochrome CYP3A and therefore require Ritonavir during their administration, to slow down their disposal. The tests were performed using human liver microsomes in the absence of Ritonavir as a booster. The results are reported in previous Table 1.

As it can be seen the metabolic stability data of **19a** are generally in line with those reported in the literature [12] on the commercially available inhibitors. In fact, this new compound presents an intrinsic *in vitro* clearance ranging from 26.8 to 121 μL / min / mg. On the other hand the intrinsic clearance shown by **19c** was comparable only to that reported for Darunavir (from 522 to 548 μL / min / mg), making this compound very promising.

Biology

All these new carbamate molecules were evaluated in their ability to inhibit the HIV-1 protease in mammalian cells, using a method developed by Lindsten et al.[13] and already used in our previous work.[5] Briefly, the expression of the precursor of HIV-1 protease bounded to green fluorescent protein (GFP) is toxic upon autocatalytic cleavage, determining the disappearance of the fluorescent signal. In the presence of a molecule with inhibitory activity, the signal due to the intact GFP-PR chimera becomes detectable and useful to monitor the *in vivo* inhibition of the enzyme.

To use properly the molecules, we initially evaluated their possible cytotoxicity towards HEK293 cells using the MTT dose-dependent viability assay. Our results determined a value of $CC_{50} > 60$ μM for all the inhibitors, after 24 hours of exposure to **10a-c** and **19a-c** molecules (data are reported in Supplementary Materials). Therefore, the values of inhibitors concentrations used in all subsequent assays did not compromise cell viability.

The quantitative analysis of the fluorescence signal given by the GFP-PR chimera, possibly inhibited by the **10a-c** and **19a-c** molecules, was performed by FACS analysis of the transfected cells, while the qualitative evaluation was performed by fluorescence microscopy. In detail, a weak background signal was detected in cells transfected with GFP-PR not treated with any inhibitor (Figure 5A, CTRL).

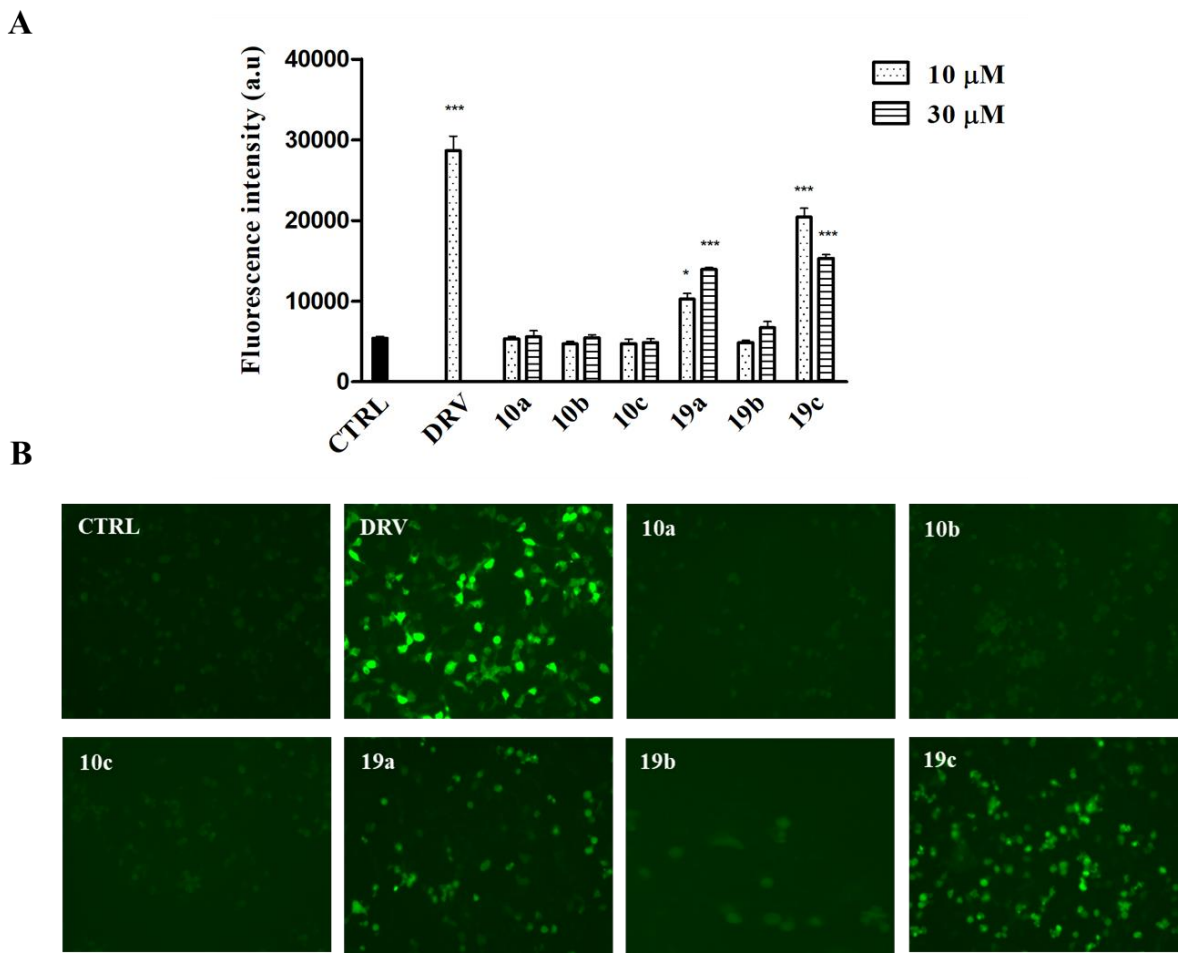


Figure 5. GFP-PR expression fluorescence analysis. **A)** *FACS analysis of HEK293 cells transiently transfected with pcDNA3/GFP-PR and treated for 24h with HIV-1 protease inhibitors at indicated concentrations. CTRL stands for not inhibited HIV-1 protease expressing cells. Data are expressed as means ± standard error (SE) of three replicates from three independent experiments and were analyzed by one-way ANOVA followed by Dunnett's post hoc test using GraphPad Prism 7 software. *p<0.05, ***p<0.001.* **B)** *HEK293 cells expressing GFP-PR fusion protein were visualized by fluorescence microscopy after incubation with 30 µM of each inhibitor (10 µ*M for **19a**) *for 24 h. CTRL stands for not inhibited HIV-1 protease expressing cells and DRV indicates 10 µM Darunavir treatment (positive control). Images (40x magnification) are representative of three independent experiments*.

In contrast, cells transfected and treated with the commercial inhibitor Darunavir (DRV) showed a strong fluorescence signal, highlighting an optimal inhibition of the viral enzyme. Cells transfected and treated with each of the putative inhibitors showed a poor fluorescence signal relatively to the molecules **10a**, **10b**, **10c** and **19b**, evaluated both at 10 µM and at 30 µM of concentration (Figure 5A), while a more than appreciable fluorescence signals were detected for the molecules **19a** and **19c**. Of note, **19a** molecule showed best activity at concentration of 30 µM, whereas **19c** compound had best inhibition activity at 10 µM. These results have been confirmed by qualitative analysis of fluorescence intensity, performed at the most active concentrations of the inhibitors (Figure 5B).

To evaluate the ability of the **19a** and **19c** molecules to be active also in the presence of some of the most common clinically significant drug-resistance mutations, we performed mutagenesis experiments on the wild type GFP-PR vector, providing the site-specific mutations V32I and

V82A.[14] As shown in figure 6A e 6B, the inhibitory activity of molecules **19a** and **19c** towards both variants of the HIV-1 protease is maintained almost unchanged compared to that shown against the wild type protease, suggesting that these mutations do not affect in any way their activity.

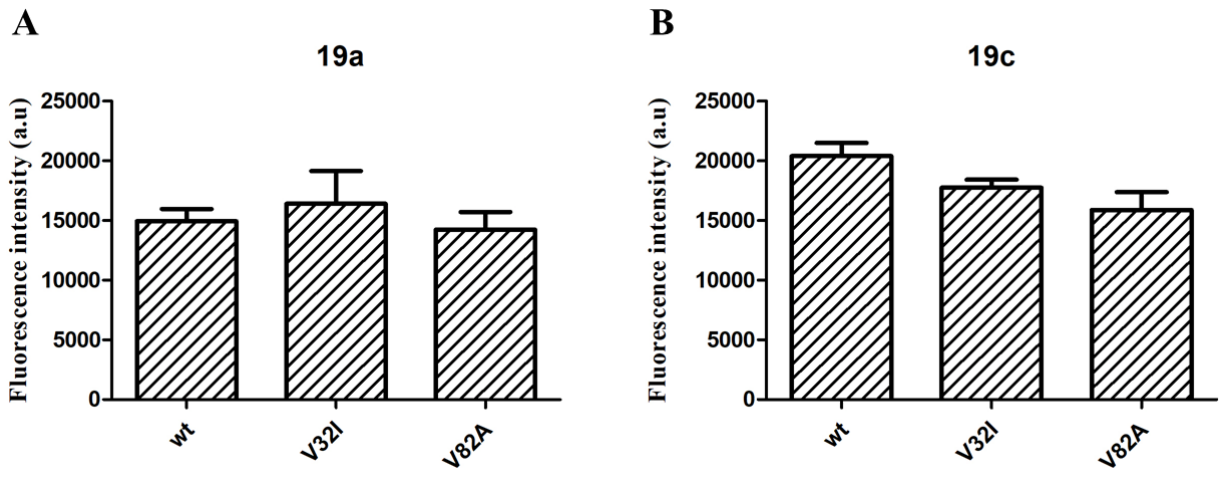


Figure 6. Evaluation of HIV-1 protease variants inhibition. *FACS analysis of HEK293 cells transiently transfected with pcDNA3/GFP-PR-V32I and with pcDNA3/GFP-PR-V82A and treated for 24h with HIV-1 protease inhibitors **A) 19a** and **B) 19c**, at 30 µM and 10 µM, respectively. Data are expressed as means ± standard error (SE) of three replicates from three independent experiments.*

3. Conclusion

In conclusion, our results showed that some of the newly synthesized molecules have inhibitory activity against HIV-1 protease in mammalian cells, confirming, moreover, the data obtained in *in vitro* assays. Although they still are less active than the commercial inhibitor Darunavir, however, maintain this activity also towards variants of the protease.

Furthermore, we can firstly highlighted that the presence of carbamate function makes these new compounds metabolically as stable as the most currently used inhibitors. In particular, benzofuran is apparently the heterocycle which confers greater metabolic stability.

Considering the excellent biological activity, the very low cytotoxicity and the good clearance profile, compounds **19a** and **19c** represent very promising molecules. Being on track to counteract drug resistance encourages us to continue our studies, optimizing the chemical structure of HIV protease inhibitors to improve their properties as much as possible.

Experimental section

Chemistry

Preparative chromatography was carried out on Merck silica gel (0.063–0.200 mm particle size) by progressive elution with opportune solvent mixtures. ^{1}H and ^{13}C NMR spectra were normally

carried out in $CDCl_3$ solutions on a VARIAN INOVA 500 MHz or Bruker 400 MHz and referenced to $CDCl_3$. Mass spectra were obtained with a Hewlett–Packard 5971 mass-selective detector on a Hewlett–Packard 5890 gas chromatograph [(OV-1 capillary column between 70 and 250 °C (20 °C min^{-1})]. The optical purity was evaluated by using a polarimeter JASCO Mod Dip-370. CH_2Cl_2 was dried by distillation over anhydrous $CaCl_2$ in inert atmosphere. Dry THF and DMF were commercially available.

(2*R*)-3-Isobutylamino-propane-1,2-diol (5)

i-$BuNH_2$ (8.0 mL, 80 mmol) was added to a stirred solution of (*S*)-glycidol **4** (0.308 g, 4.0 mmol) in *i*-PrOH (20 mL). The mixture was stirred at room temperature and after 24 h. Then the solvent and the excess of *i*-$BuNH_2$ were removed under reduced pressure. The aminodiol **5** was obtained as oil in quantitative yield. ^1H and ^{13}C NMR spectra were consistent to literature data.[4a]

(2*R*)-4-Methoxy-benzenesulfonic acid 2-hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl ester (6).

To a stirred solution of aminodiol **5** (0.115 g, 0.78 mmol) in anhydrous CH_2Cl_2 (40 mL), Et_3N (0.28 mL, 2.02 mmol) and 4-methoxybenzenesulfonyl chloride (0.384 g, 1.86 mmol) were added at room temperature and under Ar atmosphere. After 24 h the reaction was quenched with H_2SO_4 5% solution. Organic phase was washed adding saturated aqueous $NaHCO_3$ solution and brine. The organic phases were collected, dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude containing compound **6** was used in the subsequent reaction without any purification.

(2*S*)-*N*-(3-Azido-2-hydroxy-propyl)-*N*-isobutyl-4-methoxy-benzenesulfonamide (7).

The product **6** (0.78 mmol) was dissolved in anhydrous DMF (10 mL) and NaN_3 (0.104 g, 1.60 mmol) was added at room temperarure and under Ar atmosphere. The reaction mixture was warmed at reflux and kept stirring for 4 h. The reaction was then quenched by adding H_2O (20 mL). The mixture was then extracted with CH_2Cl_2 (3x30 mL) and the organic layer was dried with anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude was purified on silica gel (CH_2Cl_2/EtOAc 98:2) and compound **7** was isolated as brown oil (0.219 g, 82% from **5**).

$[\alpha]^{20}_D$ = -8.0 (c 0.5, CH_2Cl_2)

^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.73 (d, *J* = 6.8 Hz, 2H), 6.90 (d, *J* = 6.8 Hz, 2H), 4.03-3.92 (m, 1H), 3.86 (s, 3H), 3.41-3.23 (m, 3H), 3.18-2.88 (m, 4H), 1.95-1.88 (m, 1H), 0.92 (d, *J* = 5.6 Hz, 3 H), 0.85 (d, *J* = 5.6 Hz, 3H).

MS (m/z): 343 (MH^+). Anal. Calcd. for $C_{14}H_{22}N_4O_4S$: C, 49.11; H, 6.48; N, 16.36; O, 18.69; S, 9.36. Found C, 49.0; H, 6.5; N, 16.4; S, 9.4.

(2*S*)-*N*-(3-Amino-2-hydroxy-propyl)-*N*-isobutyl-4-methoxy-benzenesulfonamide (8).

Pd/C 10% (0.026 g) was added to a solution of compound **7** (0.171 g, 0.50 mmol) in EtOH (10 mL). The reaction mixture was stirred under H_2 atmosphere at room temperature. After 4 h the reaction mixture was fluxed with Ar, filtered on a Celite path, washed with EtOH and concentrated *in vacuo*. The crude containing compound **8** was used in the subsequent reaction without any purification.

$[\alpha]^{20}_D$ = -6.0 (c 0.5, CH_2Cl_2)

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.76 (d, J = 8.4 Hz, 2H), 6.99 (d, J = 8.4 Hz, 2H), 4.70 (s, 2H), 4.10 (s, 1H), 3.88 (s, 3H), 3.79 (m, 1H), 3.38-3.36 (m, 2H), 3.10-2.80 (m, 2H), 2.74 (m, 2H), 1.64 (m, 1H), 0.91 (d, J = 6.8 Hz, 3 H), 0.84 (d, J = 6.8 Hz, 3H).

MS (m/z): 317 (MH^+). Anal. Calcd. for $C_{14}H_{24}N_2O_4S$: C, 53.14; H, 7.65; N, 8.85; O, 20.23; S, 10.13. Found C, 53.0; H, 7.4; N, 8.7; S, 9.8.

Preparation of carbamates: general procedure.

Et_3N (0.033 mL, 0.24 mmol) and *p*-nitrophenylchloroformiate (0.048 g, 0.24 mmol) were added to a solution of 5-hydroxyheteroarenes **9a-c** (0.24 mmol) in anhydrous CH_2Cl_2 (1 mL), under Ar atmosphere. The mixture was kept stirring at room temperature for 1 h to afford intermediates **11a-c**; then amine **8** (or **18**) (0.20 mmol) was added and the mixture was kept stirring for 24h. The solvent was evaporated and the crude compounds were purified on silica gel (EP/EtOAc 97:3), affording to compounds **10a-c** (or **19a-c**).

(2*S*)-{2-Hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid benzofuran-5-yl ester (10a).

The product **10a** was obtained as white solid (74% yield). $[\alpha]^{20}_D$ = +10.7 (c 0.5, CH_2Cl_2).^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.75 (d, J = 8.8 Hz, 2H), 7.64 (d, J = 2.0 Hz, 1H), 7.46 (d, J = 8.8 Hz, 1H), 7.35 (d, J = 2.0 Hz, 1H), 7.04 (dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz, 1H), 6.99 (d, J = 8.8 Hz, 2H), 6.74 (brs, 1H), 3.99 (bs, 1H), 3.86 (s, 3H), 3.60 (m, 2H), 3.35 (m, 1H), 3.14 (A of ABX system, J_{AB} = 15.1 Hz, J_{AX} = 4.8 Hz, 1H), 3.04 (B of ABX system, J_{AB} = 15.1 Hz, J_{BX} = 7.3 Hz, 1H), 2.90 (d, J = 7.8 Hz, 2H), 1.88 (m, 1H), 0.93 (A of AB system, J_{AB} = 6.8 Hz, 3H), 0.92 (B of AB system, J_{AB} = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 163.1, 156.5, 152.4, 146.5, 146.3, 146.1, 129.6, 129.6, 129.4, 127.9, 118.2, 114.4, 113.7, 111.6, 106.8, 69.9, 59.1, 55.5, 53.1, 44.1, 27.19, 20.1,

19.8. MS (*m/z*): 477 (MH^+). Anal. Calcd for $C_{23}H_{28}N_2O_7S$: C, 57.97; H, 5.92; N, 5.88; O, 23.50; S, 6.73. Found C, 57.8; H, 5.7; N, 5.9; S, 6.6.

(2*S*)-{2-Hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid benzo[b]thiophen-5-yl ester (10b).

The product **10b** was obtained as white solid (72% yield). $[\alpha]^{20}_D$ = 4.5 (c 0.5, CH_2Cl_2).^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.84 (d, J = 8.6 Hz, 1H), 7.76 (d, J = 8.8 Hz, 2H), 7.59 (d, J = 2.1 Hz, 1H), 7.49 (d, J = 5.4 Hz, 1H), 7.29 (d, J = 5.4 Hz, 1H), 7.13 (dd, J_1 = 8.8 Hz, J_2 = 2.1 Hz, 1H), 6.99 (d, J = 8.8 Hz, 2H), 5.81 (bs, 1H), 4.01 (bs, 1H), 3.87 (s, 3H), 3.61 (m, 2H), 3.36 (m, 1H), 3.14 (A of ABX system, J_{AB} = 15.1 Hz, J_{AX} = 4.9 Hz, 1H), 3.05 (B of ABX system, J_{AB} = 15.1 Hz, J_{BX} = 7.2 Hz, 1H), 2.90 (d, J = 7.9 Hz, 2H), 1.89 (m, 1H), 1.63 (bs, 1H), 0.93 (A of AB system, J_{AB} = 6.8 Hz, 3H), 0.92 (B of AB system, J_{AB} = 6.8 Hz, 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 163.1, 156.3, 148.3, 140.3, 136.7, 129.7, 129.5, 128.1, 123.7, 122.9, 118.7, 115.9, 114.4, 69.9, 59.1, 55.6, 53.2, 44.2, 27.4, 20.1.MS (*m/z*): 493 (MH^+). Anal. Calcd for $C_{23}H_{28}N_2O_6S_2$: C, 56.08; H, 5.73; N, 5.69; O, 19.49; S, 13.02. Found C, 56.2; H, 5.5; N, 5.7; S, 13.2.

(2*S*)-{2-Hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid 1H-indol-5-yl ester (10c).

The product **10c** was obtained as white solid (72% yield).$[\alpha]^{20}_D$ = 32.4 (c 0.5, CH_2Cl_2).^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.32 (bs, 1H), 7.76 (d, J = 8.6 Hz, 2H), 7.37 (bs, 1H), 7.32 (d, J = 8.5 Hz, 1H), 7.22 (bs, 1H), 7.00 (d, J = 8.6 Hz, 2H), 6.95 (d, J = 8.5 Hz, 1H), 6.52 (bs, 1H), 5.76 (bs, 1H), 3.98 (bs, 1H), 3.86 (s, 3H), 3.60 (m, 1H), 3.35 (m, 1H), 3.16 (A of ABX system, J_{AB} = 15.1 Hz, J_{AX} = 4.9 Hz, 1H), 3.04 (B of ABX system, J_{AB} = 15.1 Hz, J_{AX} = 7.4 Hz, 1H), 2.91 (d, J = 7.4 Hz, 2H), 2.18 (s, 3H), 1.89 (m, 1H), 0.94 (A of AB system, J_{AB} = 5.9 Hz, 3H), 0.92 (B of AB system, J_{AB} = 5.9 Hz, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 163.1, 157.1, 144.6, 133.6, 129.7, 129.5, 128.1, 125.5, 116.3, 114.4, 112.7, 111.3, 102.9, 70.0, 59.1, 55.6, 53.2, 44.2, 27.4, 20.0.
MS (*m/z*): 476 (MH^+). Anal. Calcd for $C_{23}H_{29}N_3O_6S$: C, 58.09; H, 6.15; N, 8.84; O, 20.19; S, 6.74. Found C, 58.2; H, 6.2; N, 8.9; S, 6.7.

(1*R*,2*S*)- (1-Benzyl-2-hydroxy-3-isobutylamino-propyl)-carbamic acid tert-butyl ester (16)

i-$BuNH_2$ (8.0 mL, 80 mmol) was added to a stirred solution of (2*S*,3*S*)-1,2-epoxy-3-(Boc-amino)-4-phenylbutane **15** (1.053 g, 4.0 mmol) in *i*-PrOH (20 mL). The mixture was warmed at 60°C. After 6h the solvent and the excess of *i*-$BuNH_2$ were removed under reduced pressure. The product **16**

was obtained as white solid in quantitative yield. ^{1}H and ^{13}C NMR spectra were consistent to literature data.[8]

(1*S*,2*R*)-{1-Benzyl-2-hydroxy-3-[*iso*-butyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid tert-butyl ester (17).

To a stirred solution of aminoalcohol **16** (0.262 g, 0.78 mmol) in anhydrous CH_2Cl_2 (40 mL), Et_3N (0.28 mL, 2.02 mmol) and 4-methoxybenzenesulfonyl chloride (0.192 g, 0.93 mmol) were added at room temperature and under Ar atmosphere. After 24 h the reaction was quenched with 5% aqueous H_2SO_4 solution. The organic layer was washed adding saturated aqueous $NaHCO_3$ solution and brine. The organic phases collected were dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude was purified on silica gel (CH_2Cl_2/EtOAc 98:2) and compound **17** was isolated as white solid. ^{1}H and ^{13}C NMR spectra were consistent to literature data.[8]

(2*R*,3*S*) *N*-(3-Amino-2-hydroxy-4-phenyl-butyl)-*N*-isobutyl-4-methoxy-benzenesulfonamide (18).

To a stirred solution of **17** (0.395 g, 0.78 mmol) in anhydrous CH_2Cl_2 (29 mL), trifluoroacetic acid (13 mL) was added at room temperature. After 1 h the reaction mixture was concentrated, treated with toluene (3x20 mL) and evaporated under vacuum. The crude was purified on silica gel ($CHCl_3$/CH_3OH = 9:1) and the product **18** was obtained as white solid in 84% yield.
^{1}H and ^{13}C NMR spectra were consistent to literature data.[8]

(1*S*,2*R*)-{1-Benzyl-2-hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid benzofuran-5-yl ester (19a)

Following the general procedure the compound **19a** was obtained as white solid (75% yield). $[\alpha]^{20}_{D}$ = 17.5 (c 0.5, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.65 (d, J = 8.7 Hz, 2H), 7.55 (d, J = 2.4 Hz, 1H), 7.34 (d, J = 8.8 Hz, 1H), 7.25 (m, 4H), 7.20 (m, 1H), 7.11 (bs, 1H), 6.90 (d, J = 8.7 Hz, 2H), 6.80 (d, J = 8.8 Hz, 1H), 6.64 (bs, 1H), 5.14 (d, J = 8.3 Hz, 1H), 3.87 (bs, 2H), 3.78 (s, 3H), 3.74 (bs, 1H), 3.09 (dd, J_1 = 15.1 Hz, J_2 = 8.3 Hz, 1H), 3.01 (m, 2H), 2.92 (m, 2H), 2.75 (dd, J_1 = 13.6 Hz, J_2 = 6.8 Hz, 1H), 1.79 (m, 1H), 1.18 (bs, 1H), 0.87 (d, J = 6.5 Hz, 3H), 0.82 (d, J = 6.5 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 163.1, 155.2, 152.4, 146.4, 146.2, 137.5, 129.7, 129.6, 129.5, 128.7, 127.9, 126.7, 118.1, 114.4, 113.6, 111.6, 106.8, 72.5, 58.9, 55.6, 55.2, 53.8, 35.3, 27.3, 20.2, 19.9. MS (*m/z*): 567 (MH^+). Anal. Calcd for $C_{30}H_{34}N_2O_7S$: C, 63.59; H, 6.05; N, 4.94; O, 19.76; S, 5.66. Found C, 63.7; H, 6.1; N, 4.8; S, 5.6.

(1*S*,2*R*)-{1-Benzyl-2-hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid benzo[b]thiophen-5-yl ester (19b).

Following the general procedure the compound **19b** was obtained as white solid (71% yield). $[\alpha]^{20}_{D}$ = 14.2 (c 0.5, CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.78 (d, J = 8.5 Hz, 1H), 7.73 (d, J = 8.6 Hz, 2H), 7.48 (d, J = 5.6 Hz, 1H), 7.42 (bs, 1H), 7.32 (m, 6H), 6.98 (d, J = 8.6 Hz, 2H), 6.96 (d, J = 7.1 Hz, 1H), 5.40 (bs, 1H), 3.98 (bs, 2H), 3.85 (s, 3H), 3.14 (m, 3H), 3.00 (m, 2H), 2.85 (dd, J_1 = 13.4 Hz, J_2 = 6.7 Hz, 1H), 1.88 (m, 1H), 1.76 (bs, 1H), 0.95 (d, J = 6.5 Hz, 3H), 0.91 (d, J = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm):163.1, 154.9, 148.0, 140.1, 137.5, 136.6, 129.7, 129.5, 129.4, 128.6, 128.0, 126.6, 123.6, 122.8, 118.6, 115.8, 114.3, 72.5, 58.7, 55.6, 55.3, 53.7, 35.3, 27.2, 20.1, 19.9. MS (*m/z*): 583 (MH^+). Anal. Calcd for $C_{30}H_{34}N_2O_6S_2$: C, 61.83; H, 5.88; N, 4.81; O, 16.47; S, 11.01. Found C, 61.9; H, 5.7; N, 4.8; S, 11.1.

(1*S*,2*R*)-{1-Benzyl-2-hydroxy-3-[isobutyl-(4-methoxy-benzenesulfonyl)-amino]-propyl}-carbamic acid 1H-indol-5-yl ester (19c).

Following the general procedure the compound **19c** was obtained as white solid (70% yield).$[\alpha]^{20}_{D}$ = -18.2 (c 1.0, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 8.13 (bs, 1H), 7.65 (d, J = 8.8 Hz, 2H), 7.36 (m, 4H), 7.20 (m, 3H), 7.13 (m, 2H), 6.89 (d, J = 8.8 Hz, 2H), 6.72 (d, J = 8.5 Hz, 1H), 6.42 (bs, 1H), 5.09 (bd, J = 7.8 Hz, 1H), 3.85 (bs, 2H), 3.78 (s, 3H), 3.04 (m, 3H), 2.90 (m, 2H), 2.76 (dd, J_1 = 13.4 Hz, J_2 = 6.5 Hz, 1H), 1.80 (m, 1H), 0.86 (d, J = 6.5 Hz, 3H), 0.83 (d, J = 6.5 Hz, 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 163.0, 155.8, 144.4, 137.5, 133.5, 129.8, 129.6, 129.5, 128.6, 128.1, 126.6, 125.5, 116.2, 114.4, 112.7, 111.2, 102.9, 72.6, 55.6, 55.3, 53.8, 35.3, 27.3, 20.0, 19.8.MS (*m/z*): 566 (MH^+). Anal. Calcd for $C_{30}H_{35}N_2O_7S$: C, 63.70; H, 6.24; N, 7.43; O, 16.97; S, 5.67. Found C, 63.8; H, 6.1; N, 7.4; S, 5.6.

Modeling

The docking runs were carried out on the reference pdb structure 3NU3, complex of wild type HIV protease with amprenavir. The ligand and water molecules were removed from the structure, while hydrogens and charges were added with MGLTools. The inhibitors molecules were prepared and optimized with the MMFF forcefield as implemented in Spartan 10 (Wave Function Inc.). The size of the docking grid was 65 x 50 x 40 Å, comprising the whole protein. MD refinement of the complexes was carried out at 300 °K in the NTV ensemble with 500 ps runs.

Biology

Dulbecco's Modified Eagle Medium (DMEM), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Darunavir were purchased from Sigma Aldrich (Milan, Italy). Trypsin-EDTA solution, Fetal Bovine Serum (FBS), glutamine, penicillin-streptomycin and Phosphate Buffered Saline (PBS) were purchased from Euroclone (Milan, Italy). QuikChange XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies.

Cell culture and drug treatment

Human embryonic kidney (HEK293) cells were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. HIV-protease inhibitors were dissolved in DMSO to a stock concentration of 30 mM and diluted with complete DMEM to get the required concentrations. The final DMSO concentration in the cell cultures was no greater than 0.8%: this concentration does not affect cell viability. DMSO treated cells were used as control in all the experiments.

Cell viability analysis

Cell viability was determined considering that cells with active metabolism are able to convert MTT, a water-soluble tetrazolium salt, into a purple colored formazan product with an absorbance maximum near 570 nm[15]. Died cells lose the ability to convert MTT into formazan, thus color formation serves as a useful marker of only the viable cells. Briefly, HEK293 cells were plated in 96-well plates at a density of 1 x 10^4 cells/well and treated for 24 h with various concentrations of each HIV-1 protease inhibitor (12.5, 25, 50 and 100 μM). Cells were then incubated with MTT solution (0.5 mg/ml in DMEM) for 4 h at 37°C. The formazan crystals were finally dissolved in DMSO : isopropanol (1:1) solution. The absorbance was measured at a wavelength of 570 nm, with background subtraction at 630–690 nm, using a GLOMAX Multidetection System.

Generation of HIV-1 protease variants

HIV-1 protease mutants (V32I and V82A) were generated with QuikChange XL Site-Directed Mutagenesis Kit, according to the manufacturer's instructions, using pcDNA3-GFP-PR as a template[16]. Mutagenic primers were designed using Quick Change Primer Design Program available online at www.agilent.com/genomics/qcpd/ and each mutation was verified by sequencing.

Cell Transfection, Fluorescence microscopy and flow cytometric analysis

Transfection experiments in HEK293 cells were performed, as previously described[5], by cationic lipid transfection reagent (Trans-IT 2020, Mirus) using 0.5 μg of the expression vectors pcDNA3-GFP-PR (gift from Nico Dantuma, Addgene plasmid # 20253) and V32I and V82A variants. Cells were collected 24 hours after transfection. Where indicated, immediately after transfection, cells were treated with 10 μM or 30 μM of each HIV-1 protease inhibitor. Darunavir (DRV) 10 μM was used as positive control. For fluorescence microscopy, cells were grown on coverslips, fixed with 4% paraformaldehyde in PBS and observed under a fluorescent microscope (Nikon 80i). Analysis of GFP fluorescence was carried out resuspending cells in PBS and detecting signal using a FACS Canto II flow cytometer (Ex/Em: 480/510 nm).

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