Article

Novel Wild Type and Mutate HIV-1 Protease Inhibitors Containing Heteroaryl Carboxamides in P2: Synthesis, Biological and ADME Evaluations

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Novel Wild Type and Mutate HIV-1 Protease Inhibitors Containing Heteroaryl Carboxamides in P2: Synthesis, Biological and ADME Evaluations

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Abstract: The Virus HIV-1 infection still represents a serious disease even if actually it is transformed in chronic pathology. Considering the crucial role of the enzyme Protease in life cycle of HIV many efforts have been made in the research of new organic compounds showing inhibitory activity. After development of several series of non peptidic inhibitors we report here the synthesis of novel simple HIV-Protease inhibitors containing heteroaryl carboxamides and their antiviral activity *in vitro* and in HEK293 cells. Benzofuryl-benzothienyl- and indolyl rings as well as aryl sulfonamides with different electronic properties have been introduced by efficient synthetic procedures. All compounds showed inhibitory activity similar to the commercial drug Darunavir, effective against both wild-type HIV-1 protease and that containing the V32I or V82A mutations. ADME properties were also evaluated, showing the potential of such compounds to be developed as drugs.

Keywords: mutate HIV protease inhibitors; carboxamides; heteroarenes; mammalian cells essay; ADME

1. Introduction

In the last two decades great efforts have been dedicated to the treatment of human immunodeficiency virus (HIV-1) infection, transforming it from a fatal disease into a manageable chronic pathology. Nevertheless, over the last three years, the multiple and overlapping world crises have had a devastating impact on people living with and affected by HIV, and they have knocked back the global response to the AIDS pandemic [1].

HIV-1 Protease (HIV-1 PR) is essential to the life cycle of the virus and many inhibitors have been developed and introduced into combination-therapy regimens. Taking advantage of the detailed structure of HIV protease and its substrate, many commercially available drugs have been based on the tetrahedral transition state mimetic concept, in which a not hydrolysable hydroxyethylamine moiety has been used as the central core of the molecule [2,3].

Despite the commercial Protease Inhibitors (PIs) have proved their role in the major advances in HIV/AIDS therapies, there are still many drawbacks, mainly in terms of toxicity and systemic

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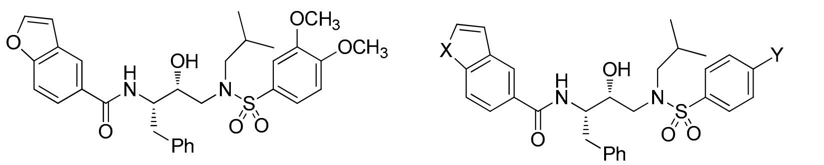
complications involving several organs [4]. Moreover, the emergence of multiple drug resistance mutations remains a challenge [5,6], reducing long-term viral inhibition.

During our investigation on new peptidomimetics and non peptidic inhibitors, we found beneficial effect of heteroaryl rings. In this respect we successfully modified first generation PIs nelfinavir and saquinavir preparing new thienyl analogues which showed the same or even higher activity against both wild type and mutant HIV protease [7].

Recently, the concept of targeting the protein backbone in structure-based drug design prompted the preparation of new non-peptidic templates, which can maximize interactions in the HIV-protease active site, particularly with the enzyme backbone atoms. This approach has led to many potent inhibitors such as FDA-approved Darunavir and several related compounds [8–13].

Following this concept, we developed a systematic study on simple substituted stereodefined isopropanolamine derivatives, in which the high effect of the moiety between the heteroaryl group and the *core*, as well as the type of heteroaryl group, were evident [14–16]. Easily synthesized benzothienyl-, benzofuryl- and indolyl derivatives bearing either a carboxyamide or a carbamoyl spacer in general showed high *in vitro* activity against native protease. They also confirmed their inhibition activity in mammalian cells, showing a general low citotoxicity and great metabolic stability, thus demonstrating their promising potential [17–20].

In particular, regarding carboxyamide derivatives we found critical the presence of benzyl fragment in the *core*, reaching an IC_{50} value of 1 nM *in vitro* for compound **A** (Figure 1)[17]. Hence, with the aim of checking the effect of different arylsulfonamide groups (in terms of electronic properties) and heteroaryl rings as P2 ligand, we run a systematic study on the synthesis and on the inhibition activity of new simple derivatives of structure **B**.



A; IC_{50} = 1nM　　**B**　　X = O, NH, S; Y = OCH_3, NO_2

Figure 1. Active carboxamide inhibitor **A** and new simple derivatives **B.**

2. Materials and Methods

2.1. Chemistry

Preparative chromatography was carried out on Merck silica gel (0.063–0.200 mm particle size) by progressive elution with suitable solvent mixtures. ^1H and ^{13}C NMR spectra were 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 rotation was evaluated by using a polarimeter JASCO Mod Dip-370. CH_2Cl_2 was dried by distillation over anhydrous $CaCl_2$ in an inert atmosphere. Dry THF and DMF were commercially available,

All ^1H and ^{13}C NMR spectra for the following compounds were consistent to literature data: (1*R*,2*S*)-(1-Benzyl-2-hydroxy-3-*iso*-butylamino-propyl)-carbamic acid *tert*-butyl ester (2) [17], (1*S*,2*R*)-[1-Benzyl-2-hydroxy-3-*iso*-butyl-(4-methoxy-benzenesulfonyl)amino-propyl]-carbamic acid *tert*-butyl ester (3a) [18], (1*S*,2*R*)-[1-Benzyl-2-hydroxy-3-*iso*-butyl-(4-nitro-benzenesulfonyl)amino-propyl]-carbamic acid *tert*-butyl ester (3b) [21], (2*R*,3*S*)-*N*-(3-Amino-2-hydroxy-4-phenyl-butyl)-*N*-isobutyl-4-methoxy-benzenesulfonamide (4a) [18], (2*R*,3*S*)-*N*-(3-Amino-2-hydroxy-4-phenyl-butyl)-*N*-isobutyl-4-nitro-benzenesulfonamide (4b) [20].

General procedure for carboxyamides **5a-c** and **6a-c**

To a solution of 5-heteroaryl acid (0.13 mmol), EDCI (0.20 mmol), HOBt (0.20 mmol) in anhydrous CH_2Cl_2, a solution of amine **4a-b** (0.13 mmol) and diisopropylethylamine (0.78 mmol) in anhydrous CH_2Cl_2 was added at 0°C under argon atmosphere and it was allowed to stir for 16h at

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room temperature. The reaction mixture was quenched with water and extracted with CH_2Cl_2. The organic layers were dried on Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (CH_2Cl_2/AcOEt 9/1) to furnish **5a-c** and **6 a-c** .

***N*-((2*S*,3*R*)-3-hydroxy-4-(N-*iso*butyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl)benzo[b]thiophene -5-carboxamide (5a).** Following the general procedure compound **5a** was obtained as a white solid, in 42% yield. $[\alpha]^{D}_{20}$=+3.7 (c 0.2, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09 (s, 1H), 7.89 (d, *J* = 8.4 Hz, 1H), 7.68 (d, *J* = 8.8 Hz, 2H), 7.55 (m, 2H), 7.30 (m, 6H), 6.93 (d, *J* = 8.8 Hz, 2H), 6.55 (d, *J* = 8.4 Hz, 1H), 4.44 (m, 2H), 4.03 (m, 1H), 3.85 (s, 3H), 3.17 (m, 4H), 2.89 (m, 2H), 1.88 (m, 1H), 0.89 (d, *J* = 6.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.3, 163.0, 142.9, 139.4, 137.8, 130.3, 129.8, 129.4, 128.7, 128.0 126.7, 124.2, 122.7, 122.6, 122.3, 114.3, 72.9, 58.9, 55.6, 54.7, 53.6, 35.0, 27.2, 20.1, 20.0.

***N*-((2*S*,3*R*)-3-hydroxy-4-(N-*iso*butyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl)benzo[b]furan-5-carboxamide (5b).** Following the general procedure, the compound **5b** was obtained as a white solid, in 44% yield.$[\alpha]^{D}_{20}$=+9.6 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.67 (m, 3H), 7.56 (d, *J* = 8.6 Hz, 1H), 7.47 (d, *J* = 8.6Hz, 1H), 7.28 (m, 4H), 7.22 (m, 1H), 6.91 (d, *J* = 8.8 Hz, 2H), 6.78 (brs, 1H), 6.58 (d, *J* = 8.0 Hz, 1H), 4.48 (brs, 1H), 4.42 (m, 1H), 4.05 (m, 1H), 3.84 (s, 3H), 3.16 (m, 5H), 2.88 (m, 2H), 1.88 (m, 1H), 0.87 (d, *J* = 6.5 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.3, 163.0, 156.6, 146.3, 137.9, 129.8, 129.41, 129.40, 129.1, 128.6, 127.5, 126.6, 123.3, 120.7, 114.2, 111.4, 106.9, 72.9, 58.8, 55.6, 54.7, 53.5, 35.0, 27.2, 20.1, 20.0

***N*-((2*S*,3*R*)-3-hydroxy-4-(N-*iso*-butyl-4-methoxyphenylsulfonamido)-1-phenylbutan-2-yl)indol-5-carboxamide (5c).** Following the general procedure, the compound **5c** was obtained as a white solid, in 45% yield. $[\alpha]^{D}_{20}$=+22.2° (c 1.1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.94 (d, *J* = 13.0 Hz, 1H), 7.94 (s, 1H), 7.66 (d, *J* = 8.7 Hz, 2H), 7.43 (d, *J* = 8.6 Hz, 1H), 7.28 (m, 7H), 6.88 (d, *J* = 8.7 Hz, 2H), 6.55 (brs, 2H), 4.69 (brs, 1H), 4.40 (m, 1H), 4.04 (m, 1H), 3.81 (s, 3H), 3.31 (dd, *J* = 15.2 Hz, *J* = 4.8 Hz, 1H), 3.15 (m, 2H), 3.05 (dd, *J* = 15.2 Hz, *J* = 7.6 Hz, 1H), 2.92 (dd, *J* = 13.3 Hz, *J* = 7.2 Hz, 1H), 2.82 (dd, *J* = 13.3 Hz, *J* = 7.2 Hz, 1H), 1.88 (m, 1H), 0.85 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.5, 162.9, 138.0, 137.7, 129.7, 129.5, 129.4, 128.6, 127.5, 126.6, 126.0, 125.4, 120.7, 120.3, 114.3, 111.2, 103.4, 73.0, 58.8, 55.6, 54.9, 53.5, 35.1, 27.2, 20.1, 20.0.

***N*-((2*S*,3*R*)-3-hydroxy-4-(N-*iso*-butyl-4-nitrophenylsulfonamido)-1-phenylbutan-2-yl)benzo[b]thiophene-5-carboxamide (6a).** Following the general procedure, the compound **6a** was obtained as a white solid, in 54 % yield. $[\alpha]^{D}_{20}$= +6.8 (c 0.3, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 8.30 (d, *J* = 8.6 Hz, 2H), 8.09 (s, 1H), 8.03 (brs, 1H), 7.92 (d, *J* = 8.6 Hz, 2H), 7.83 (d, *J* = 8.8 Hz, 1H), 7.53 (m, 2H), 7.36 (m, 1H), 7.30 (m, 5H), 4.35 (m, 1H), 4.02 (m, 1H), 3.35 (brd, *J* = 15.2 Hz, 1H), 3.19 (m, 1H), 3.13 (brd, *J* = 7.1 Hz, 2H), 3.00 (m, 2H), 1.91 (m, 1H), 0.86 (d, *J* = 5.7 Hz, 3H), 0.85 (d, *J* = 5.7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 169.7, 150.0, 145.1, 137.7, 137.21, 135.1, 129.3, 128.8 (3C), 128.4, 128.3, 126.8, 125.8, 124.3, 120.9, 120.4, 71.8, 55.1, 53.4, 51.3, 35.6, 31.4, 30.2, 29.7

***N*-((2*S*,3*R*)-3-hydroxy-4-(N-*iso*-butyl-4-nitrophenylsulfonamido)-1-phenylbutan-2-yl)benzofuran-5-carboxamide (6b).** Following the general procedure, the compound **6b** was obtained as a white solid, in 56% yield. $[\alpha]^{D}_{20}$= -3.0° (c 0.2, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (d, *J* =8.8 Hz, 2H), 7.92 (d, *J* =8.8 Hz, 2H), 7.87 (s, 1H), 7.69 (s,1H), 7.53 (A part of AB system, J_{AB} =8.8 Hz, 1H), 7.49 (B part of AB system, J_{AB} = 8.8 Hz, 1H), 7.32 (m, 4H), 6.81 (s, 1H), 6.41 (d, *J* = 7.5 Hz, 1H), 4.45 (brs, 1H), 4.34 (brs, 1H), 4.02 (brs, 1H), 3.35 (brd, *J* = 15.2 Hz, 1H), 3.20 (dd, *J* = 15.2Hz, *J* = 8.4 Hz, 1H), 3.13 (d, *J* = 6.8 Hz, 2H), 3.03 (dd, *J* = 13.7, *J* = 7.2 Hz, 1H), 2.95 (dd, *J* = 13.7, *J* = 7.2 Hz, 1H), 1.91 (m, 1H), 0.87 (d, *J* = 6.8 Hz, 3H), 0.85 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.7, 156.7, 150.0, 146.5, 144.7, 137.5, 129.3, 129.2, 128.8, 128.5, 127.6, 126.9, 124.3, 123.2, 120.7, 111.5, 106.9, 72.4, 57.7, 55.3, 52.5, 35.2, 26.9, 19.9.

***N*-((2*S*,3*R*)-3-hydroxy-4-(N-*iso*-butyl-4-nitrophenylsulfonamido)-1-phenylbutan-2-yl)indol-5-carboxamide (6c).** Following the general procedure, the compound **6c** was obtained as a white solid, in 83% yield.$[\alpha]^{D}_{20}$= + 26.7 (c 1.2, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.66 (brs, 1H), 8.22 (d, *J* =8.8 Hz, 2H), 7.89 (s, 1H), 7.88 (d, *J* =8.8 Hz, 2H), 7..44 (d , J = 8.6 Hz, 1H), 7.34 (d, *J* = 8.6 Hz, 1H) 7.31 (m, 4H), 7.26 (m, 2H), 6.58 (brs, 1H), 6.43 (d, *J* = 7.4 Hz, 1H), 4.35 (m, 1H), 4.00 (m, 1H), 3.39 (dd, *J* = 15.1 Hz, *J* = 4.0 Hz, 1H), 3.19 (dd, *J* = 15.1 Hz, *J* = 8.4 Hz, 1H), 3.13 (d, *J* = 7.1 Hz, 2H), 3.07 (dd, *J* = 13.6

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Hz, *J* = 8.0 Hz, 1H), 2.92 (dd, *J* = 13.6 Hz, *J* = 7.2 Hz, 1H), 1.91 (m, 1H), 0.87 (d, *J* = 6.8 Hz, 3H), 0.84 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.7, 149.8, 144.7, 137.7, 137.5, 129.3, 128.8, 128.5, 127.5, 126.9, 125.9, 125.2, 124.2, 120.8, 120.3, 111.2, 103.7, 72.4, 57.6, 55.4, 52.3, 35.5, 26.9, 19.9.

2.2. Biology

2.2.1. Materials

Dulbecco's Modification of Eagle's Medium (DMEM) was purchased from Corning. Dimethyl sulfoxide (DMSO), Trypsin–EDTA solution and Darunavir were purchased from Sigma Aldrich-Merck. Dulbecco's Phosphate Buffered Saline (DPBS), L–Glutamine, Penicillin-Streptomycin solution and Fetal Bovine Serum were obtained from EuroClone. Lipofectamine 3000 was purchased from Thermofisher.

2.2.2. Cell culture and drug treatment

Human embryonic kidney (HEK293) cells were cultured 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 solubilized in DMSO as a 5 mM stock solution and then diluted in complete DMEM to 10 μM as working concentration. Cells treated only with 0.2% DMSO (vehicle) were used as control.

2.2.3. Cell transfection and FACS analysis

HEK293 cells were seeded in 24-well plates at the density of 2.5×10^5 cells/well. After 24 hours, cells were transfected with pcDNA3/GFP-PR plasmid (gift from Nico Dantuma, Addgene plasmid # 20253) or with mutant plasmids carrying V32I or V82A mutation, generated as previously described [18], using Lipofectamine 3000 according to the manufacturer's instructions.

Where indicated, immediately after transfection, cells were treated with HIV-1 protease inhibitors at 10 μM. Cells treated with Darunavir (DRV) were used as positive control. GFP fluorescence was quantified in HEK293 cells harvested 24 hours after trasfection and resuspended in DPBS, using a BD FACS Canto II flow cytometer (Ex/Em: 480/510 nm) [17].

2.2.4. Statistical analysis

Data were presented as means ± Standard Error (SE) of three independent experiments, each performed in triplicate and were analyzed by GraphPad Prism software (version 8, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test (p-values <0.05 were considered as statistically significant).

3. Results

3.1. Chemistry

The synthetic approach for the preparation of compounds of general structure **B** is well established [17] and uses a four step reactions sequence (Scheme 1).

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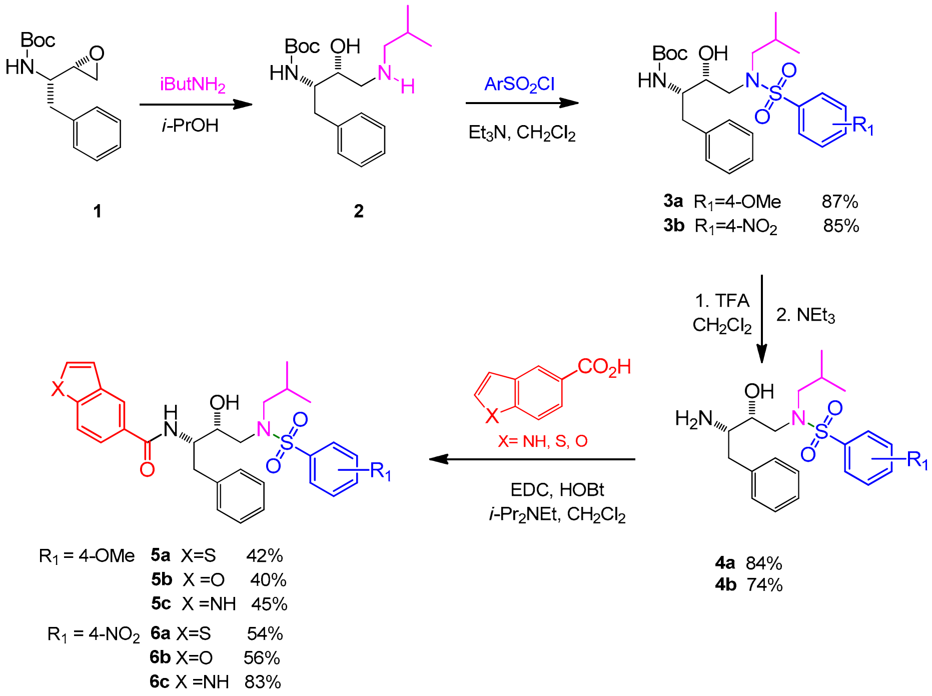


Scheme 1. Preparation of compounds **5** and **6.**

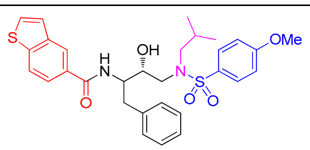
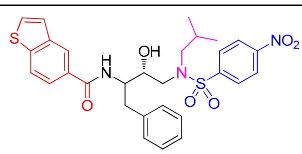
In particular, if a benzyl group is present in the central *core* (Figure 1), synthesis started from commercially available homochiral *N*-Boc protected amino epoxide **1** (Scheme 1). The epoxide was firstly opened with *iso*-butyl amine to afford the monoprotected diaminoalcohol **2**, 4-OMe- and 4-NO_2-phenylsulfonyl moieties were alternatively introduced on secondary amine, affording **3a** and **3b**, respectively. *N*-Boc group was then efficiently displaced by TFA and the crude ammonium trifluoroacetate derivatives were treated with Et_3N, affording the free amines **4a-b** [18,20].

From these common intermediates, we were easily able to achieve two class of compounds: **5a-c** and **6a-c**, in which heteroaryl group is spaced from *core* by carboxyamide functionality. This was inserted reacting amines **4a-b** with the suitable 5-heteroarylcarboxylic acid, previously activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt).

3.2. Biology

All these new compounds were tested as HIV Protease inhibitors firstly *in vitro* against the wild type enzyme and the results are reported in Table 1. All the tested compounds were powerful inhibitors of the native enzyme *in vitro*. The IC_{50} of three derivatives, namely **5a**, **5b** and **6a**, could not be measured under our experimental conditions, as the enzyme was fully inhibited even at 0.1 nM concentration of the inhibitors. However, also **5c**, **6b** and **6c** were extremely active, with IC_{50} ranging from 0.6 to 95 nM.

Table 1. Structure and activity of compounds **5a-c** and **6a-c.**

Entry	Inhibitor	IC_{50} (nM)	Entry	Inhibitor	IC_{50} (nM)
1	 **5a**	< 0.6	4	 **6a**	< 0.6

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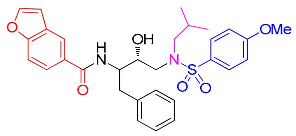
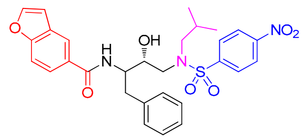
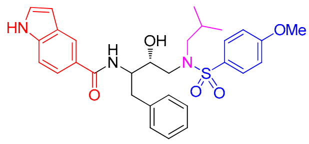
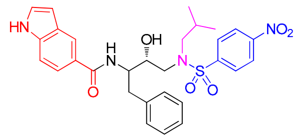
2	 **5b**	< 0.6 n	5	 **6b**	4.0 ± 0.5
3	 **5c**	95 ± 7	6	 **6c**	0.60 ± 0.03

The ability of these compounds to inhibit HIV-protease in HEK293 cells was also evaluated, using the assay developed by Lindsten et al. [22], as described in our previous work [17,18].

Briefly, HEK293 cells transiently expressing a nontoxic HIV-protease precursor fused to GFP protein were treated with 10 μM of each inhibitor and GFP fluorescence was quantified by FACS.

As shown in Figure 2, cells treated without any inhibitor showed a low fluorescence, because the recombinant protein is toxic by autocatalytic cleavage; conversely, in presence of proper inhibitors, cells were able to express intact chimera and GFP fluorescence accordingly increased. The tested compounds (**5a, 5b, 5c, 6a, 6b, 6c**) were able to inhibit wild type HIV-1 protease (Figure 2A) similarly to Darunavir (no significant differences were detected), encouraging to test their inhibitory capacity also towards HIV-protease carrying mutation V32I or V82A.

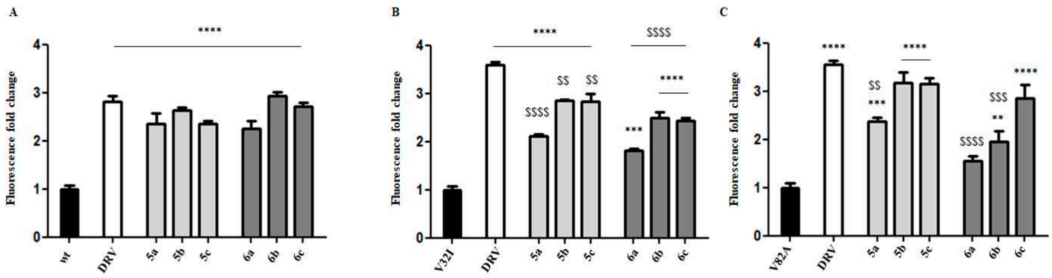


Figure 2. Evaluation of HIV-1 protease inhibition. HIV-1 protease activity was detected measuring GFP fluorescence in HEK293 cells transiently transfected with pcDNA3/GFP-PR (wild type, wt) or with recombinant plasmids, expressing HIV-protease carrying mutation V32I or V82A, and treated for 24h with 10 μM HIV-1 protease inhibitors (**5a, 5b, 5c, 6a, 6b, 6c**). In each panel, the cells transfected with the plasmid (wt, V32I or V82A) and untreated with any inhibitor are to be considered as negative controls; cells transfected with the indicated plasmid and exposed to DRV are to be considered as positive controls. Relative fluorescence is expressed as fold change respect to transfected cells cultured without any inhibitor. All data are expressed as means ± Standard Error (SE) of three experiments, each performed in triplicate and statistical significance was evaluated using GraphPad Prism 8.4.2 software by one-way ANOVA followed by Dunnett's post hoc test, **p <0.01, ***p <0.001, ****p<0.0001 versus control [respectively wt (A), V32I (B) or V82A (C)]; $$p<0.01, $$$p <0.001, $$$$p<0.0001 versus DRV.

All compounds inhibited the viral protease carrying V32I mutation, although less efficiently than commercial inhibitor used as positive control, as demonstrated by significant differences revealed between our molecules and Darunavir (Figure 2B).

As shown in Figure 2C, cells transfected with mutant plasmid carrying V82A mutation and treated with **6a** showed poor fluorescence, comparable to fluorescence measured in control cells, suggesting that the V82A mutation compromised **6a** inhibitory activity. Compounds **5a** and **6b** were able to inhibit mutant protease, but the levels of fluorescence detected were lower than those

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measured in presence of Darunavir; conversely, significant accumulation of GFP reporter was detected in cells treated with **5b**, **5c** and **6c** (no significant differences were revealed respect to Darunavir), indicating that V82A mutation do not affect in any way their activity.

4. Discussion

This paper is the last of a long series, as our work in the design, synthesis and optimization of inhibitors bearing heterocyclic systems at P1/P2 positions started in 2012 and has led to over 30 selected molecules tested on the wt HIVpro [14,15,17–19,23–26].

During our research, we started from initial IC_{50} values of ten millimolar obtained for a few molecules among large pools of inactive compounds and arrived at pools containing only active molecules with sub-nanomolar affinities.

In our previous papers, we have carried out extensive molecular modelling to explain the observed structure-activity relationships and we have discussed in detail the modelled interactions involving indole and benzofuran side chains, and the eventual entropic effects connected to less counterproductive desolvation in benzothiophene. This analysis has been helpful to drive our design to more focused structures, and in the first works we have been able to correct the most unfavorable features of the first sets of inhibitors (such as the length of the chain connecting the interacting groups as in the case of carbamates) [27].

The most powerful inhibitors found in our previous works are reported for reference in Figure 3.

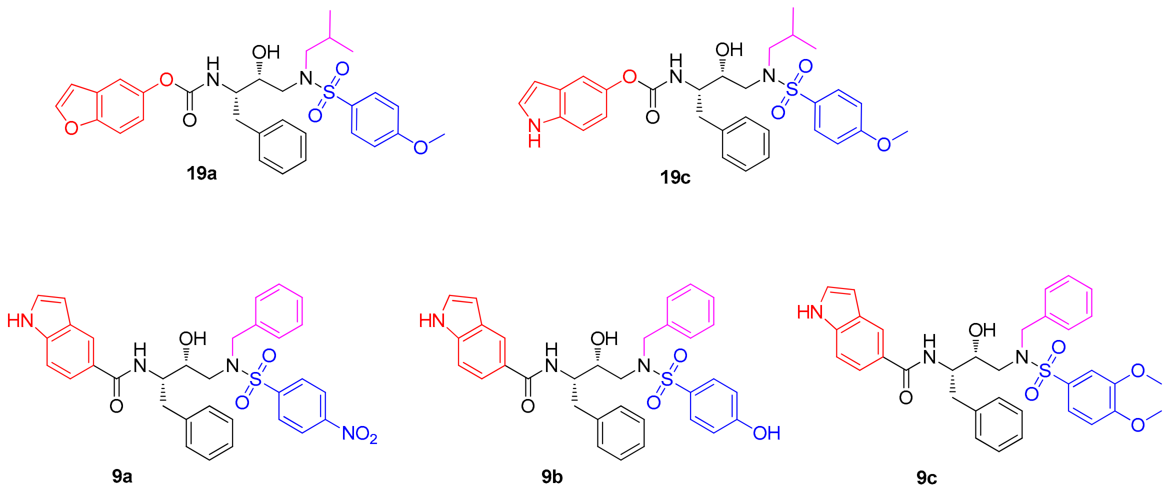

Figure 3. inhibitors with IC_{50} < 0.6 nM described in our previous works; compounds are numbered according to the original schemes in refs. [18] and [19].

With the reported molecules in this paper, and those reported in the previous ones we reached a limit in which most of the molecules show IC_{50} values that are under the limit of measure of our experimental systems (< 0.6 nM, as we work at 1.2 nM enzyme). In this condition, molecular modelling is no longer useful to obtain further insights in the binding mode of the inhibitors, as we cannot compare calculated energies or scores with experimental values.

Thus, rather than report modeling explanations for the results found in this paper, we attempted to carry out an overall analysis of the results of the whole of our work.

We have selected a set of the 27 best inhibitors from this and our previous works, with affinities ranging from 15 μM to < 0.6 nM. (Figure S1) and we have calculated the values of commonly used molecular descriptors for all the compounds, including molar refractivity, total polar surface area, logP, logS (water solubility), log Kp (skin permeability) (see Table S2) [28] .

We have then carried out a covariance analysis of our experimental log (IC_{50}) and the descriptors, and we have found that activity is slightly (but suggestively) correlated directly with logP (0.758) and inversely with molar refractivity and logS (-0.771, -0.757). This suggests that hydrophobic interactions are more important than polar ones to increase the affinity for the catalytic site.

Such outcome is not unexpected due to the nature of the HIVpr site, however it seems to confirm that we have driven hydrophobic interactions to a limit.

We have also carried out an evaluation of the ADME properties in comparison with the predicted properties of Darunavir on the SwissADME facility: the results are reported in Table 2S, where compounds performing better or equally than Darunavir in several rule systems are highlighted in green, while worse compounds are highlighted in red. 12 out 27 compounds (including **5b** and **5c** described in this work) perform better or equally than Darunavir in all the ADME prediction models. The greater inhibitory activity of **5b** and **5c** molecules, respect to other molecules tested, was also highlighted in the assays conducted in HEK293 cells. The efficacy of these compounds was comparable to that of darunavir both towards HIV-1 protease wild type and towards V82A mutated variant. Mutation V32I, instead, seems to weakly compromise their activity.

5. Conclusions

In conclusion, in this work we have reported the synthesis of a novel series of carboxamidic compounds with a high inhibitory activity against both the native HIV-1 protease and a mutant one; the inhibitory activity has been measured both *in vitro* and in mammalian cells, by using methodologies just reported in our previous papers. The obtained results can open new perspectives in the research for new inhibitors to overcome the problem of drug resistance. Furthermore, the ADME evaluations show that not only the novel inhibitors reported in this paper, but all the compounds in the **Table 1** are competitive with Darunavir structure.

Work is in progress in the attempt to understand the structure-activity relationships for a develop of the research.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Structures of the inhibitors used in the analysis of ADME properties; Table S1: Collection of molecular descriptors for the set of inhibitors; Figure S2: ADME predictions; Copies of ^{1}H and ^{13}C NMR spectra of compounds **5a-c** and **6a-c** recorded in $CDCl_3$ with a Varian 400 MZ instrument.

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