Bioorganic & Medicinal Chemistry xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Design, synthesis and preliminary SAR studies of novel *N*-arylmethyl substituted piperidine-linked aniline derivatives as potent HIV-1 NNRTIs

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ARTICLE INFO

Article history:
Received 26 September 2013
Revised 21 October 2013
Accepted 22 October 2013
Available online xxxx

Keywords:
HIV-1
AIDS
NNRTIs
Synthesis
Anti-HIV-1 activity

ABSTRACT

A series of novel *N*-arylmethyl substituted piperidine-linked aniline derivatives were designed, synthesized and evaluated for their anti-HIV activity in MT-4 cells. All the new compounds showed moderate to potent activities against wild-type (wt) HIV-1 with an EC_{50} range from 0.022 to 2.1 μM. Among them, compound **5a6** (EC_{50} = 0.022 ± 0.0091 μM, SI >10,770) was confirmed to be the most potent and selective inhibitor, which proved more potent than DDI and DLV in a cell-based assay against wt HIV-1, and more efficient than NVP in an RT (reverse transcriptase) assay. Besides, it is worth noting that compound **7a1** retained moderate inhibitory activity (EC_{50} = 4.8 ± 0.95 μM) against the HIV-1 double RT mutant strain (K103N/Y181C). The preliminary structure–activity relationship was discussed and rationalized by molecular simulation.

1. Introduction

The number of people living with human immunodeficiency virus (HIV) has been rising steadily in the past two decades. According to the latest statistic data[1] provided by WHO, 34.0 million people were living with HIV and 1.7 million people died from AIDS in 2011, globally. Fortunately, the current coverage of highly active anti-retroviral therapy (HAART) has distinctly deferred people dying from AIDS-related causes. In the combinatory regimens of HAART, nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs), identified as a chemically diverse class of compounds targeting at an allosteric binding pocket on HIV-1 RT have gained a definitive place because of their high potency, low toxicity and impressive selectivity against HIV-1 replication.[2] However, the high mutation rate of RT has promoted the selection of drug-resistant virus which have significantly hindered the continued successful antiretroviral therapy.[3,4] Therefore, the development of novel NNRTIs with an improved activity profile against drug-resistant HIV mutants is continuously needed.

Investigations focused on the resistance profiles of NNRTIs for almost two decades gave rise to the discovery and development of diarylpyrimidine (DAPY) derivatives (Fig. 1), such as the clinically approved drugs, etravirine (TMC125) and rilpivirine (TMC278), which retain activity against a large panel of HIV-1 mutant strains.[5,6] Whereas a defect in bioavailability of most DAPY derivatives resulted from their low water solubility has provided much of the motivation for further structural optimization based on this chemical skeleton.[7] Subsequent modification using the principles of molecular hybridization and bioisosteric replacement led to the discovery of piperidine-linked aminopyrimidine derivatives and diarylaniline derivatives, respectively, which displayed broad spectrum against both wild-type (wt) and drug-resistant viral strains.[8,9] In our previous work,[7,10] a series of piperidine-linked triazine derivatives were designed, synthesized and evaluated for anti-HIV activities, and several compounds revealed excellent potencies with EC_{50} values in low nanomolar concentration range against the wt HIV-1 strain and in low micromolar concentration range against the frequently encountered HIV-1 double mutant strain K103N/Y181C.

Promoted by these results and in continuation of our study, we designed a novel series of *N*-arylmethyl substituted piperidine-linked aniline derivatives by utilizing the molecular hybridization principle. In the newly designed compounds (Fig. 2), the 4-cyano/methyl-2,6-dimethylphenoxy, which was assumed to be a preponderant group in the left wing of TMC120 or TMC125, was adopted

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http://dx.doi.org/10.1016/j.bmc.2013.10.033

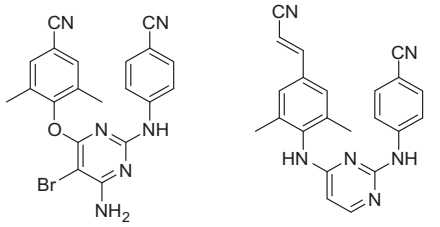


Figure 1. Structures of clinically used DAPY derivatives.

to accommodate the hydrophobic sub-pocket of RT. And a nitro-, amino- or amide substituted benzene, which was confirmed to be an acceptable bioisostere of pyrimidine ring, was used as the core ring and was expected to generate a hydrogen bond with the amino acid of RT. Moreover, inspired by the drug design strategy that forming additional protein–inhibitor interactions, especially a bidentate hydrogen-bond between the inhibitor and the amino acid main chain may prevent resistance development,[11] the *N*-arylmethyl substituted piperidine in the right wing, previously reported to make multiple hydrogen bonds with the backbone NH of K103 through a water bridge,[8] was employed for improving the resistance profile. Herein, we report the synthesis, anti-HIV activities of these newly designed compounds, as well as their preliminary structure–activity relationship (SAR) analysis.

2. Results and discussion

2.1. Chemistry

A modified synthetic route to afford the target products **5a1**–**5a10**, **5b1**–**5b10**, **7a1** and **11** is shown in Scheme 1. Treatment of the initial material 4-chloro-2-fluoro-1-nitrobenzene (**1**) with *tert*-butyl 4-aminopiperidine-1-carboxylate gave intermediate **2**,[12,13] which was reacted with corresponding substituted phenols in the catalysis of KI and PTC (phase transfer catalyst) at a high temperature to afford the key intermediates **3a** and **3b** in excellent yields. Deprotections of **3a** and **3b** in an easy, rapid and mild condition provided **4a** and **4b**, which were sufficiently pure for the following reactions without further purification.[10,14–16] Subsequently, *N*-alkylation reactions of **4a** or **4b** with appropriately substituted arylmethyl halides achieved the final compounds **5a1**–**5a10** and **5b1**–**5b10**.[10] Reduction of the nitrobenzene compound where Ar was 4-sulfanilylphenyl (**5a8**) gave the intermediate **6a1**, which afforded the desired compound **7a1** after acidification.[17] In another route, **3a** was reduced by hydrogenation, and then acetylated to give the intermediate **9**, which was followed by deprotection, affording the intermediate **10**. The target product **11** was finally obtained by an alkylation reaction of compound **10** with 4-(bromomethyl)benzenesulfonamide. In addition, the substituted arylmethyl halides were commercially available or prepared according to similar methods reported in the literatures.[18–22] Both analytical and spectral data of all the compounds are in full agreement with the proposed structures.

2.2. Biological activities

2.2.1. *In vitro* anti-HIV assay

The compounds were evaluated for their *in vitro* anti-HIV activity in MT-4 cells infected by wt HIV-1 strain III_B,[23] commonly encountered double RT mutant HIV-1 strain RES056 (K103N + Y181C), and HIV-2 strain ROD. The cytotoxicity of the compounds was also tested in parallel in HIV-uninfected MT-4 cells. The results, interpreted as EC_{50}, CC_{50}, SI (selectivity index) and fold resistance, are summarized in Table 1 with the marketed drugs zidovudine (azidothymidine, AZT), dideoxyinosine (DDI), DMP266 (efavirenz, EFV) and PNU-90152T (delavirdine, DLV) as the reference standards.[24,25]

As shown in Table 1, it was gratifying to see that all the synthesized compounds were superior to the reference drug DDI against wt HIV-1(III_B) with the exception of **11**, which was totally inactive. Half of them were more potent than the positive control drug DLV. The active derivatives exhibited moderate to high activity against the wt HIV-1 strain III_B with EC_{50} values ranging from 0.022 to 2.1 μM and SI values in the variable range of 4 to >10,770. Of all the evaluated derivatives, compound **5a6** emerged as the most potent and selective inhibitor against wt HIV-1, possessing a low EC_{50} value of 0.022 ± 0.0091 μM and a high SI of >10,770, which

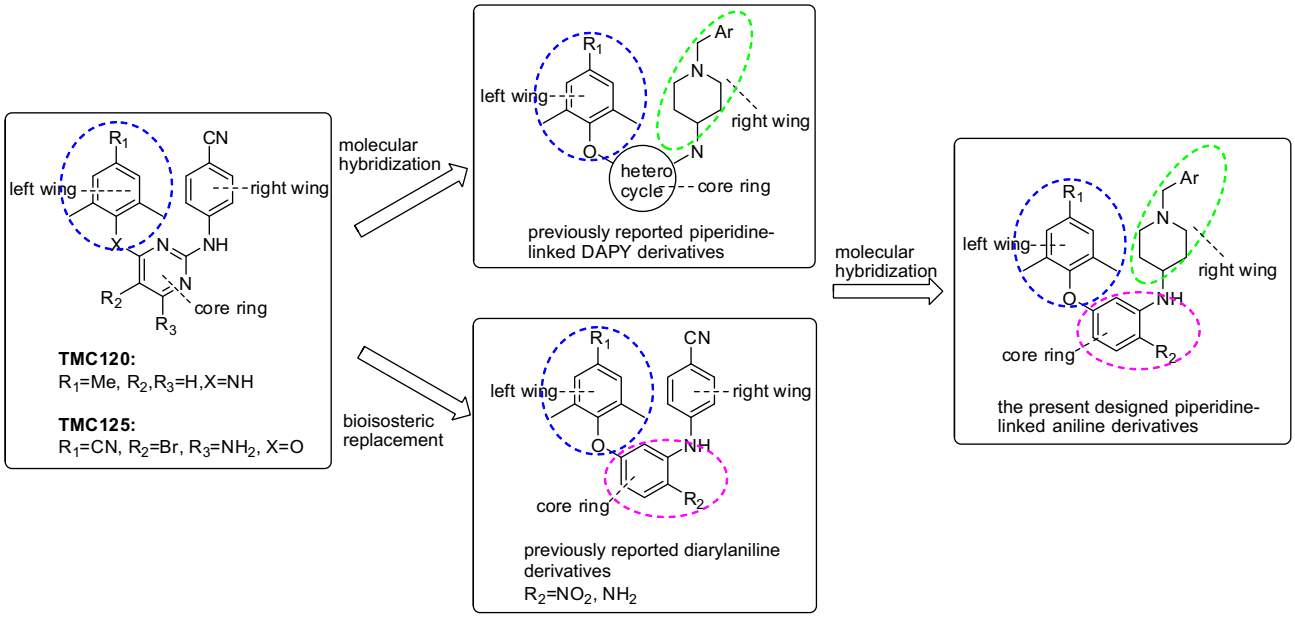
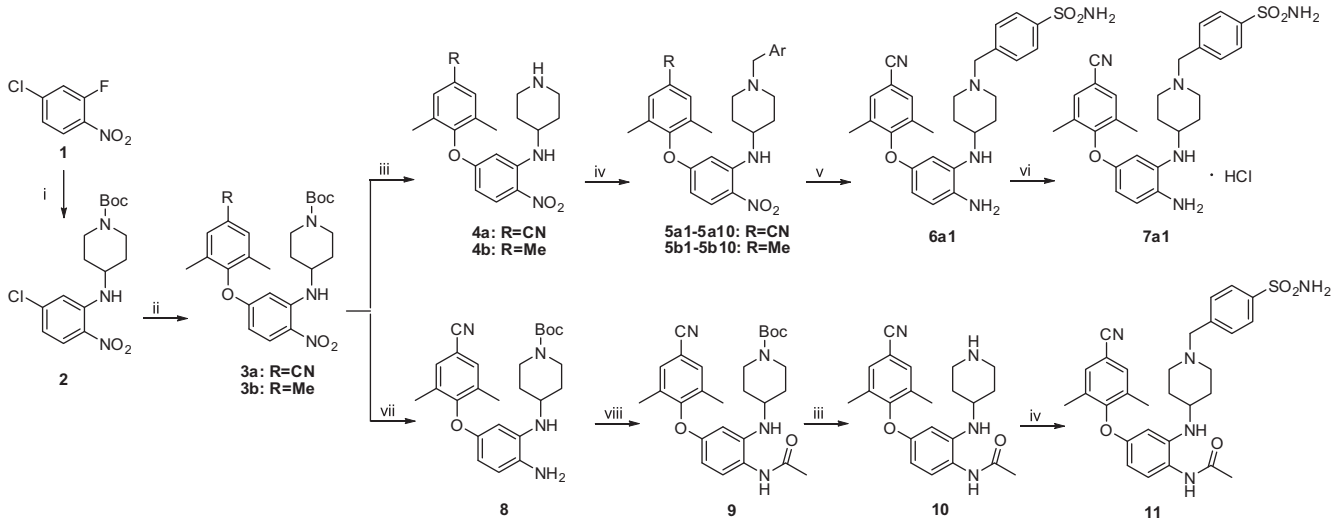


Figure 2. Newly designed piperidine-linked aniline derivatives.



Scheme 1. The synthetic route of target compounds. Reagents and conditions: (i) *tert*-butyl 4-aminopiperidine-1-carboxylate, Na_2CO_3, DMF, 80 °C, 1 h; (ii) substituted phenol, K_2CO_3, TBAB, KI, DMF, 120–130 °C, 12 h; (iii) TFA, DCM, rt, 20 min; (iv) K_2CO_3, DMF, rt; (v) Pd/C, H_2, THF, EtOH, rt, 36 h; (vi) con. hydrochloric acid, DCM, 0 °C; (vii) Pd/C, H_2, THF, EtOH, rt, overnight; (viii) acetyl chloride, TEA, DCM, 0 °C, 1 h.

attained the same order of magnitude as those obtained for the reference drugs AZT and EFV.

These analogues were also assayed against the frequently encountered HIV-1 double mutant strain K103N/Y181C (RES056). Both the absolute activity against the HIV-1 mutant (EC_{50} value) and the relative activity (fold-resistance) were used to define the resistance profile of the tested compounds. As shown in Table 1, compound **7a1** was identified as the unique active compound which was more potent than the reference drug DLV against the resistant mutant strain with an EC_{50} value of 4.8 ± 0.95 μM (*versus* >36 μM, DLV) and a fold resistance ratio of 114 (*versus* >277, DLV). In addition, all the title compounds were screened for their inhibition against the replication of the HIV-2 strain (ROD), but none of them exhibited inhibitory activity at sub-toxic concentrations, indicating that the novel series of *N*-arylmethyl-substituted piperidine-linked aniline derivatives were specific to HIV-1.

First, we focused our attention on the SAR of the substituent R_1 at the left wing. When the core ring and the right wing of each compound were mutual coincide, cyan proved to be the favored group for wt HIV-1 strain inhibition compared with methyl in most instances. For example, the series **5a** (with a cyano group) showed better potency against wt HIV-1 than the series **5b** (with a methyl group), except for compounds **5a3**, **5a4** and **5a7** (*versus* **5b3**, **5b4** and **5b7**, respectively).

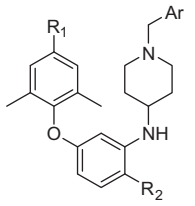
Next, the SAR of the substituted Ar of the right wing was explored. The diverse structures of this moiety produced distinct impact on the activities against the wt HIV-1. It was interesting to notice that the antiviral efficiency and selectivity index increased dramatically when the carbonyl at the *para* position of the benzyl moiety (**5a3**, **5b3**, **5a7** and **5b7**) was replaced with sulfuryl (**5a6**, **5b6**, **5a8** and **5b8**), especially for compounds **5a3** and **5b3** *versus* compounds **5a6** and **5b6**, respectively. The antiviral activity of compound **5a8**/**5b8** (with-SO_2NH_2) and the counterpart **5a6**/**5b6** (with-SO_2Me) was almost at the same level. Meanwhile, the compounds with the carbonyl in the *para* substituent displayed considerable antiviral potency, which decreased steadily in the order **5a7**/**5b7** (–$CONH_2$) >**5a3**/**5b3** (–COMe) >**5a5**/**5b5** (–COOH). These results in conjunction with previous studies[10] suggested that the sulfuryl/carbonyl group included in the *para*-substituent played a dominant role, when the substituent possessed appropriate hydrophilicity.

It was especially interesting to notice that the wt HIV-1 inhibitory activity was markedly enhanced when the unsaturated carboxyl group at the benzyl moiety was replaced by a saturated hydroxymethyl group (**5a5** *versus* **5a4**, **5b5** *versus* **5b4**), while the negative impact on the cytotoxicity was further corroborated with this replacement. This result indicated that, the unsaturated or conjugate substituent at the *para*-position proved beneficial for decreasing the cytotoxicity of the tested compound.

Furthermore, as the data demonstrated, compounds **5a1** and **5b1** with a hydrophobic substituent nitro at the *para*-tail possessed the lowest inhibitory activity against wt HIV-1 among both the series of compounds **5a** and **5b**. In the meantime, all the compounds with various hydrophilic *para*-substituents (**5a2**–**5a8**, **5b2**–**5b8**) revealed better potency in inhibiting wt HIV-1 compared with the corresponding no-*para*-substituent compounds **5a10** and **5b10**, which were endowed with the lowest selectivity indices. Practically, compound **5a7**/**5b7** with a hydrophilic *para*-carbamoyl exhibited much better antiviral activity and higher cytotoxicity than compound **5a9**/**5b9** with a hydrophobic *para*-methoxycarbonyl. Furthermore, the introduction of a pyridylmethyl moiety at the benzyl domain led to a substantial increase in the potency and selectivity against wt HIV-1, for instance, **5a2** *versus* **5a10** and **5b2** *versus* **5b10**. Thus, the results led us to hypothesize that a *para* substituent at the benzyl moiety is crucial for the selectivity index of the tested compound and a hydrophilic group accommodates the chemical environment well in this region of RT, which is grossly in accordance with results of the previously reported piperidine-linked triazine series.[7,10]

We also investigated the SAR of the substituent R_2 at the core ring. Obviously, the results revealed that the anti-HIV activity of compounds **5a1**–**5a10** and **5b1**–**5b10** bearing a NO_2 substituent is limited to the wt HIV-1, irregardless the substitution pattern. Nevertheless, compound **7a1** ($R_2 = NH_2$) retained high activity against the wt HIV-1 and exhibited considerable inhibitory activity against a resistant mutant RT strain of HIV-1 (K103N/Y181C) without augmenting the cytotoxicity compared to compound **5a8** ($R_2 = NO_2$). However, compound **11**, which introduced an acetyl at the NH_2 group in the case of compound **7a1**, displayed no activity against both wt HIV-1 and HIV-2 strains. It can be concluded that the NH_2 at the core ring was probably beneficial for the binding affinity (potentially hydrogen bonding interaction) between

Table 1
Anti-HIV-1 activity and cytotoxicity of the newly synthesized congeners



No.	R_1	R_2	Ar	EC_{50}[a] (μM)			CC_{50}[b] (μM)	SI[c]		Fold res.[d]
				HIV-1		HIV-2		HIV-1		HIV-1
				IIIB	RES056	ROD		IIIB	RES056	RES056
5a1	CN	NO_2	4-Nitrophenyl	0.67 ± 0.28	>182	>182	182 ± 18	269	<1	>269
5a2	CN	NO_2	Pyridin-4-yl	0.056 ± 0.0033	>19	>19	19 ± 3.1	336	<1	>336
5a3	CN	NO_2	4-Acetylphenyl	0.15 ± 0.051	>251	>251	>251	>1630	X1	>1,630
5a4	CN	NO_2	4-(Hydroxymethyl) phenyl	0.14 ± 0.076	>11	>11	11 ± 5.9	80	<1	>80
5a5	CN	NO_2	4-Carboxylphenyl	0.19 ± 0.10	>30	>30	30 ± 1.9	159	<1	>159
5a6	CN	NO_2	4-(Methylsulfonyl) phenyl	0.022 ± 0.0091	>234	>234	>234	>10,770	X1	>10,770
5a7	CN	NO_2	4-Aminoacylphenyl	0.062 ± 0.015	>27	>27	27 ± 2.6	430	<1	>430
5a8	CN	NO_2	4-Sulfanilylphenyl	0.029 ± 0.014	>23	>23	23 ± 1.7	782	<1	>782
5a9	CN	NO_2	4-Methoxycarbonylphenyl	0.13 ± 0.041	>160	>160	⩾160	⩾1208	<orX1	>1208
5a10	CN	NO_2	Phenyl	0.63 ± 0.32	>2.8	>2.8	2.8 ± 1.6	4.4	<1	>4.4
5b1	Me	NO_2	4-Nitrophenyl	2.1 ± 0.83	>147	>147	147 ± 16	70	<1	>70
5b2	Me	NO_2	Pyridin-4-yl	0.082 ± 0.048	>21	>21	21 ± 3.6	258	<1	>258
5b3	Me	NO_2	4-Acetylphenyl	0.11 ± 0.032	>138	>138	138 ± 66	1312	<1	>1312
5b4	Me	NO_2	4-(Hydroxymethyl) phenyl	0.096 ± 0.028	>25	>25	25 ± 1.4	255	<1	>255
5b5	Me	NO_2	4-Carboxylphenyl	0.22 ± 0.049	>35	>35	35 ± 8.8	161	<1	>161
5b6	Me	NO_2	4-(Methylsulfonyl) phenyl	0.027 ± 0.014	>158	>158	158 ± 55	5895	<1	>5895
5b7	Me	NO_2	4-Aminoacylphenyl	0.059 ± 0.012	>27	>27	27 ± 2.1	457	<1	>457
5b8	Me	NO_2	4-Sulfanilylphenyl	0.036 ± 0.014	>22	>22	22 ± 2.4	615	<1	>615
5b9	Me	NO_2	4-Methoxycarbonylphenyl	1.0 ± 0.054	>248	>248	>248	>246	X1	>246
5b10	Me	NO_2	Phenyl	0.66 ± 0.36	>25	>25	25 ± 1.9	37	<1	>37
11	CN	$NHCOCH_3$	4-Sulfanilylphenyl	>228		>228	>228	X1		
7a1 (Hydrochloride)	CN	NH_2	4-Sulfanilylphenyl	0.043 ± 0.0020	4.8 ± 0.95	>22	22 ± 0.61	505	4.4	114
AZT				0.0072 ± 0.00029	0.0074 ± 0.00087	0.0057 ± 0.00050	>94	>13,066	>12,594	1.0
DDI				19 ± 2.6		18 ± 1.8	>212	>11	>12	
EFV				0.0074 ± 0.0026	0.52 ± 0.022		>6.3	>855	>12	70
DLV				0.13 ± 0.043	>36		>36	>277	X1	>277

All data were calculated using the median value of two or three parallel assays.
[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cell against HIV-induced cytotoxicity, as determined by the MTT method.
[b] CC50: concentration required to decrease the viability of mock-infected cells by 50%, as determined by the MTT method.
[c] SI: selectivity index (CC_{50}/EC_{50}). The SI values: X1 stand for ⩾1or<1.
[d] Fold res.: ratio of EC_{50} value against drug-resistant strain and EC_{50} of wt HIV-1(III_B) ($EC_{50}^{mut}/EC_{50}^{wt}$).

the inhibitor and the active binding site of the double mutant strain (K103N/Y181C), and the conjecture is also in agreement with those reported in the literatures.[9,26] Further in-depth analysis of this issue will be presented in the following section concerning molecular simulation.

In general, the SAR analysis above can be summarized as follows: (1) Substituted phenyl is an acceptable isosteric replacement for the anti-HIV-1 activity instead of heterocycles in the core ring domain. It may be noted that substitution at the R_2 position of the piperidine-linked aniline skeleton deserves further consideration towards improving the drug-resistance profile. (2) The potency and selectivity index of the compounds are strongly dependent on the nature of the substituent at the *para* position of the benzyl. (3) Appropriate hydrophilicity of the aforementioned substituent plays a significant role in the bioactivity of the compounds. (4) An unsaturated or conjugate substituent coupling directly with the *para*-benzyl is essential for decreasing the cytotoxicity, and the sulfuryl is confirmed to be a privileged group. The comprehensive SAR concluded above provides helpful information for further design and investigation of novel anti-HIV-1 analogues.

2.2.2. HIV-1 RT inhibition assay

We hypothesize the newly synthesized *N*-arylmethyl substituted piperidine-linked aniline derivatives as HIV-1 NNRTIs based on the similarity with the lead compounds. To further confirm their binding target, we selected the most active compound **5a6** for the RT enzyme assay, utilizing poly(A) × $oligo(dT)_{15}$ as the template primer. As the data indicated (Table 2), compound **5a6** was more potent than the typical NNRTI NVP (IC_{50} = 0.67 μM) with an IC_{50} value of 0.36 μM, while inferior to that of ETV (IC_{50} = 0.071 μM). The result manifested that these newly synthesized derivatives represented by compound **5a6** targeted the HIV-1 RT, thus acting as classical NNRTIs.

Table 2
Inhibitory activity of compound **5a6** against wt HIV-1 RT[a]

Compd	**5a6**	NVP	ETV
IC_{50}[b] (μM)	0.36	0.67	0.071

[a] The RT kit was commercially available and supplied by Roche.
[b] IC_{50} means the inhibitory concentration of the tested compounds required to inhibit biotin deoxyuridine triphosphate (biotin-dUTP) incorporation into the HIV-1 RT by 50%.

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2.3. Molecular modeling

Molecular modeling was carried out to give further insight in the probable binding modes at the allosteric binding site of RT and rationalize some SAR conclusions. More specifically, the representative compounds **5a8**, **7a1** and **11** were chosen to be docked into the nonnucleoside inhibitor binding pocket (NNIBP) of HIV-1 RT (PDB code: 3M8Q and 3BGR) in terms of Autodock Vina 1.1.2 [http://vina.scripps.edu] (Fig. 3). Default parameters were used as described in the Autodock Vina 1.1.2 manual unless otherwise specified.

As illustrated in Figure 3, compounds **5a8** and **7a1** docked into the wt RT binding pocket (PDB code: 3M8Q) displayed a similar binding mode as the lead piperidine-linked aminopyrimidine derivatives.[8,10] Specifically, the pattern can be characterized as follows: (a) The left wing of compounds **5a8** and **7a1** fit into a hydrophobic sub-pocket mainly defined by the side chains of aromatic amino acid residues Tyr181, Tyr188 and Trp229 and generated a positive π–π stacking interaction. (b) Compounds **5a8** and **7a1** reserved features of both Lys101 and Lys103 main-chain binding types, wherein the hydrogen bond between the N atom of the piperidine and the backbone of residue Lys103 was bridged by a water molecule. (c) The hydrophilic benzsulfamide tail resided between Val106 and Pro236 and was oriented to the solvent exposed region, which can explain the SAR conclusion that a hydrophilic substituent at the tail of the right wing was favored. However, the position of the left wing of compound **11** revealed a deflection in the hydrophobic sub-pocket due to the substitution of an acetamino at the core ring, and directly resulted in a decreased interaction between the inhibitor and the side chain of the Tyr181 residue as well as the lose of a water-bridged hydrogen bond. In addition, compound **11** displayed the worst overlap with the ligand, and the docking results could account for why compounds **5a8** and **7a1** exhibited similar excellent potency, whereas compound **11** showed a sharp decrease in antiviral activity.

Compound **7a1** was also simulated in a K103N/Y181C mutant RT binding pocket (PDB code: 3BGR). The result revealed that the potent antiviral compound retained the hydrogen bond between NH and Lys101. It is worth noting that a lose of a water-mediated hydrogen bond with the Lys103 backbone in wt binding mode was remedied by the hydrogen bond formed between the aniline NH_2 and the Asn103 side chain CO. Thus, the bidentate hydrogen bond was preserved in the mutant binding mode to meet the drug-resistance design strategy proposed in the design and explaining why compound **7a1** was active against the K103N/Y181C HIV-1 mutant strain.

In summary, the molecular simulation analysis conformed to the original design, rationalized the SAR conclusion above and strongly supported the bioactivity data of the newly designed and synthesized NNRTIs. Further investigations on the structural basis will take these aspects into account.

3. Conclusion

In summary, we have designed and synthesized a series of novel *N*-arylmethyl substituted piperidine-linked aniline derivatives, and evaluated their biological activity against HIV-1 (wt strain III_B and double mutant strain K103N/Y181C) and HIV-2 (strain ROD). It was gratifying to see that the majority of the title compounds exhibited high potency against HIV-1. Among the newly synthesized congeners, the most potent and selective compound against wt HIV-1 was **5a6**, which was more active than the reference drugs DDI and DLV, with an EC_{50} value of 0.022 ± 0.0091 μM and a SI value of >10,770. Besides, compound **7a1** retained moderate inhibitory activity (EC_{50} = 4.8 ± 0.95 μM) against the HIV-1 double mutant strain (K103N/Y181C). These piperidine-linked anilines could be defined as orthodox NNRTIs based on the enzyme assay and the fact that none of the congeners was shown to be an anti-HIV-2 agent. The preliminary SAR and molecular simulation were also studied, pro-

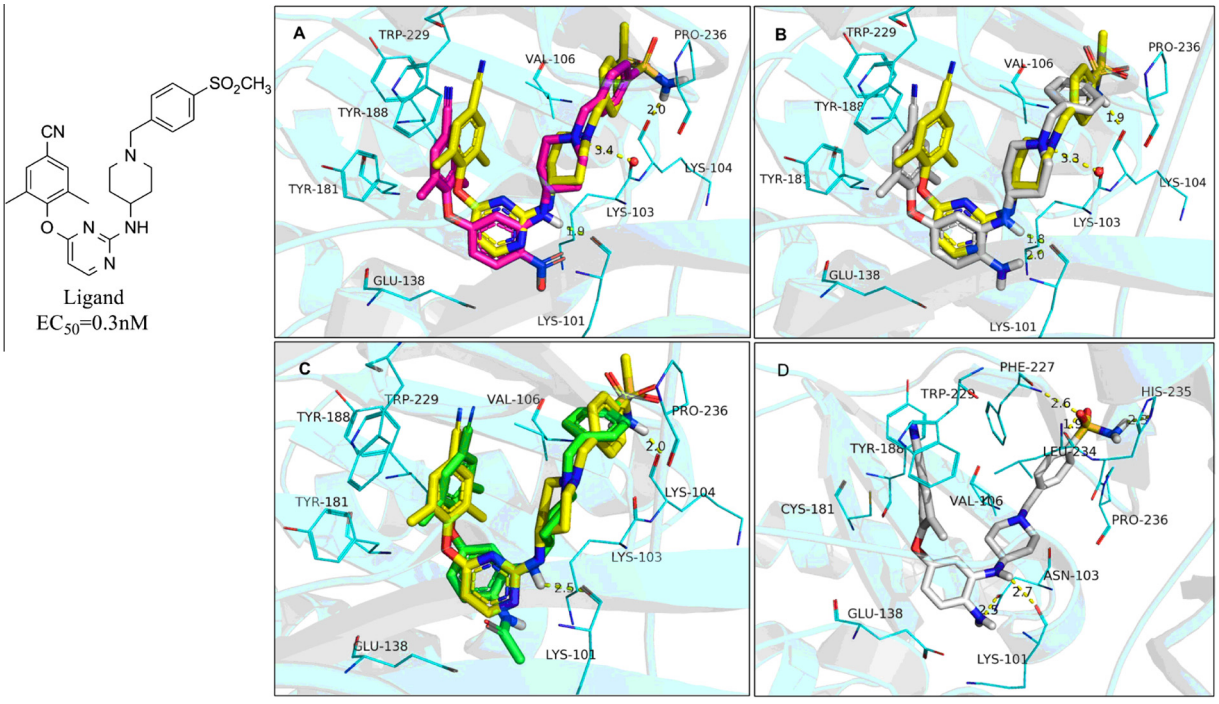

Figure 3. (A) The predicted binding mode of **5a8** (red) into wt NNIBP (PDB code: 3M8Q) and the overlap with the ligand (yellow). (B) The predicted binding mode of **7a1** (gray) into wt NNIBP (PDB code: 3M8Q) and the overlap with the ligand (yellow). (C) The predicted binding mode of **11** (green) into wt NNIBP (PDB code: 3M8Q) and the overlap with the ligand (yellow). (D) The predicted binding mode of **7a1** into double mutant (K103N/Y181C) NNIBP (PDB code: 3BGR). All the figures were generated using PyMol (www.pymol.org).

viding helpful guidance for further rational design of novel anti-HIV-1 NNRTIs.

4. Experimental section

4.1. Chemistry

All melting points were determined on a micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer in the appropriate solvents and tetramethylsilane (TMS) as internal standard. ^{13}C NMR spectra were run in the same instrument at 100 MHz. Chemical shifts are expressed in δ units and TMS as internal reference. Mass spectra were taken on a LC Autosampler Device: standard G1313A instrument (Agilent Technologies, Company). TLC was performed on Silica Gel GF254 for TLC (Merck) and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Flash column chromatography was performed on column packed with Silica Gel 60 (200–300 mesh). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of a rotary evaporator at reduced pressure.

4.1.1. General procedure for the synthesis of *tert*-butyl 4-(5-chloro-2-nitrophenylamino)piperidine-1-carboxylate (2)

A mixture of 4-chloro-2-fluoro-1-nitrobenzene (**1**, 0.09 g, 0.5 mmol), *tert*-butyl 4-aminopiperidine-1-carboxylate (0.1 g, 0.5 mmol), and sodium carbonate (0.15 g, 1.4 mmol) in DMF (5 ml) was stirred under 80 °C for 1 h. Then DMF was evaporated and the crude reaction product was dissolved in 10 ml dichloromethane (DCM) and 10 ml brine. The aqueous solution was extracted with DCM (10 ml×3). The combined organic layer was washed with brine (10 ml×3) and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed by evaporation and the residue was purified by column chromatography on silica to give the compound **2** (0.18 g, yield: 100%) as a yellow solid. Mp: 93–95 °C. ESI-MS: *m*/*z* 373.3 (M+18), 375.4 (M+2+18), 378.4 (M+23). $C_{16}H_{22}ClN_3O_4$ [355.13].

4.1.2. General procedure for the synthesis of *tert*-butyl 4-(5-(4-cyano-2,6-dimethylphenoxy)-2-nitrophenylamino)piperidine-1-carboxylate (3a)

A mixture of *tert*-butyl 4-(5-chloro-2-nitrophenylamino)piperidine-1-carboxylate (**2**, 0.1 g, 0.28 mmol), 4-cyan-3,5-dimethylphenol (0.04 g, 0.27 mmol), potassium carbonate (0.08 g, 0.58 mmol), tetrabutyl ammonium bromide (0.02 g, 0.062 mmol), potassium iodide (0.01 g, 0.060 mmol) and DMF (5 ml) as the solvent was heated to 120–130 °C and stirred for 12 h. The solvent was removed under reduced pressure and redissolved in 10 ml DCM and 10 ml brine. The liquid was separated and the water phase was extracted with DCM (10 ml×3), the organic layers were combined, washed with brine (10 ml×3) and dried over anhydrous sodium sulfate. After filtration, the solvent was removed by evaporation and the residue was purified by column chromatography on silica to give the compound **3a** (0.11 g, yield: 84.6%) as a yellow solid. Mp: 125–127 °C. ESI-MS: *m*/*z* 467.5 (M+1), 484.5 (M+18), 489.5 (M+23). $C_{25}H_{30}N_4O_5$ [466.22].

4.1.3. General procedure for the synthesis of *tert*-butyl 4-(5-(mesityloxy)-2-nitrophenylamino)piperidine-1-carboxylate (3b)

tert-Butyl 4-(5-chloro-2-nitrophenylamino)piperidine-1-carboxylate (**2**, 0.05 g, 0.14 mmol), 2,4,6-trimethylphenol (0.04 g, 0.29 mmol), potassium carbonate (0.08 g, 0.58 mmol), tetrabutyl ammonium bromide (0.01 g, 0.03 mmol), potassium iodide (0.005 g, 0.03 mmol) in DMF (1.5 ml) was stirred at 120–130 °C for 6 h. The mixture was poured into water. The aqueous was extracted with ethyl acetate for 3 times. The combined organic phase was washed with brine, dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated and the crude reaction product was purified by column chromatography on silica using petroleum ether/ethyl acetate (10:1) as eluent to give an orange oil, and then recrystallized to obtain the product **3b** (63.5 mg, yield: 99.2%) as a yellow solid. ESI-MS: *m*/*z* 456.5 (M+1), 473.3 (M+18), 478.4 (M+23). $C_{25}H_{33}N_3O_5$ [455.24].

4.1.4. General procedure for the synthesis of 3,5-dimethyl-4-(4-nitro-3-(piperidin-4-ylamino)phenoxy)benzonitrile (4a) and *N*-(5-(mesityloxy)-2-nitrophenyl)piperidin-4-amine (4b)

To a solution of *tert*-butyl 4-(5-(4-cyano-2,6-dimethylphenoxy)-2-nitrophenylamino)piperidine-1-carboxylate (**3a**, 4.00 g, 8.58 mmol) in dichloromethane (13 ml) was added trifluoroacetic acid (13 ml) dropwise for 0.5 h in a water bath at 15 °C. The reaction mixture was stirred for another 0.5 h, the water bath was replaced by an ice-bath and the saturated sodium carbonate (90 ml) was added in drops. The precipitate was filtered and dried in vacuum to give the intermediate **4a** (3.09 g, yield: 98.4%) as an orange solid. ESI-MS: *m*/*z* 367.3 (M+1), 389.4 (M+23). $C_{20}H_{22}N_4O_3$ [366.17].

The intermediate **4b** was obtained as a yellow solid (yield: 99.5%) following the procedure in the preparation of **4a**. Mp: 152–154 °C. ESI-MS: *m*/*z* 356.4 (M+1), 378.4 (M+23). $C_{20}H_{25}N_3O_3$ [355.19].

4.1.5. General procedure for the synthesis of target compounds (5a1–5a10 and 5b1–5b10)

A mixture of **4a** or **4b** (1 equiv), corresponding substituent (0.99–1.56 equiv) and potassium carbonate (2.11–3.22 equiv) in DMF was stirred at room temperature for 1.5–12 h. The reaction mixture was poured into an excess of water and filtered over celatom. The residue was dissolved with dichloromethane and dried over anhydrous sodium sulfate. The filtrate was concentrated and purified by column chromatography on silica to afford the target compounds (**5a1**–**5a10** and **5b1**–**5b10**), respectively.

4.1.5.1. 3,5-Dimethyl-4-(4-nitro-3-(1-(4-nitrobenzyl)piperidin-4-ylamino)phenoxy)benzonitrile (5a1). Elution with petroleum ether/DCM/ethyl acetate (1:1:0.1). Orange solid, yield: 100%, mp: 208–210 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.28 (d, 1H, J = 7.1 Hz, NH), 8.19 (d, 2H, J = 8.6 Hz, C_3,C_5-Ph′-H), 8.14 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.53 (d, 2H, J = 8.5 Hz, C_2,C_6-Ph′-H), 7.46 (s, 2H, C_3,C_5-Ph″-H), 6.13 (d, 1H, J = 2.4 Hz, C_3-Ph-H), 5.97 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.63 (s, 2H, CH_2), 3.36 (m, 1H, C_1-Pi-H), 2.78 (t, 2H, Pi-CH_2), 2.25 (t, 2H, Pi-CH_2), 2.17 (s, 6H, 2× CH_3), 2.00 (m, 2H, Pi-CH_2), 1.68 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 162.84 (Ph-C_4), 153.77 (Ph″-C_1), 147.21, 146.49, 146.43, 133.05, 133.01, 130.22, 129.35, 127.77, 123.59, 118.39 (C≡N), 109.79, 103.13, 97.66 (Ph-C_3), 62.07 (Pi-CH_2-Py), 51.61, 48.96, 31.52, 16.16 (2× CH_3). ESI-MS: *m*/*z* 502.3 (M+1), 524.4 (M+23), 540.4 (M+39). $C_{27}H_{27}N_5O_5$ [501.20].

4.1.5.2. 3,5-Dimethyl-4-(4-nitro-3-(1-(pyridin-4-ylmethyl)piperidin-4-ylamino)phenoxy)benzonitrile (5a2). Elution with petroleum ether/ethyl acetate/triethylamine (2:1:0.2). Yellow solid, yield: 71.0%, mp: 169–171 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.56 (dd, 2H, J_1 = 1.4 Hz, J_2 = 4.5 Hz, C_3,C_5-Py-H), 8.28 (d, J = 7.1 Hz, 1H, NH), 8.15 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.46 (s, 2H, C_3,C_5-Ph′-H), 7.30 (d, 2H, J = 5.7 Hz, C_2,C_6-Py-H), 6.12 (d, 1H, J = 2.4 Hz, C_3-Ph-H), 5.97 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.55 (s, 2H, CH_2), 3.36 (m, 1H, C_1-Pi-H), 2.79 (t, 2H, Pi-CH_2), 2.25 (t, 2H, Pi-CH_2), 2.17 (s, 6H, 2× CH_3), 2.01 (m, 2H, Pi-CH_2), 1.69 (m, 2H, Pi-CH_2). ^{13}C NMR

(100 MHz, $CDCl_3$, ppm) δ: 162.84 (Ph-C_4), 153.75 (Ph″-C_1), 149.88, 146.49, 133.04, 133.01, 130.24, 127.76, 123.76, 118.39 (C≡N), 109.79, 103.11, 97.65 (Ph-C_3), 61.63 (Pi-CH_2-Py), 51.57, 31.41, 16.18 (2× CH_3). ESI-MS: *m*/*z* 458.5 (M+1). $C_{26}H_{27}N_5O_3$ [457.21].

4.1.5.3. 4-(3-(1-(4-Acetylbenzyl)piperidin-4-ylamino)-4-nitrophenoxy)-3,5-dimethylbenzonitrile (5a3). Elution with petroleum ether/ethyl acetate/triethylamine (5:1:0.2). Yellow solid, yield: 66.2%. mp: 110–112 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.28 (d, 1H, *J* = 7.2 Hz, NH), 8.14 (d, 1H, *J* = 9.4 Hz, C_6-Ph-H), 7.93 (d, 2H, *J* = 8.2 Hz, C_3,C_5-Ph′-H), 7.45 (s, 2H, C_3,C_5-Ph″-H), 7.44 (d, 2H, *J* = 9.3 Hz, C_2,C_6-Ph′-H), 6.12 (d, 1H, *J* = 2.5 Hz, C_3-Ph-H), 5.96 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.59 (s, 2H, CH_2), 3.34 (m, 1H, C_1-Pi-H), 2.79 (t, 2H, Pi-CH_2), 2.61 (s, 3H, CO–CH_3), 2.22 (t, 2H, Pi-CH_2), 2.17 (s, 6H, 2× CH_3), 1.99 (m, 2H, Pi-CH_2), 1.68 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 197.86 (C=O), 162.81 (Ph-C_4), 153.76 (Ph″-C_1), 146.53, 144.11, 136.17, 133.04, 133.00, 130.21, 128.96, 128.44, 127.72, 118.40 (C≡N), 109.77, 103.05, 97.66 (Ph-C_3), 62.53 (Pi-CH_2-Py), 51.58, 49.10, 31.54, 26.65 (CO-CH_3), 16.17 (2× CH_3). ESI-MS: *m*/*z* 499.4 (M+1), 521.4 (M+23). $C_{29}H_{30}N_4O_4$ [498.23].

4.1.5.4. 4-(3-(1-(4-(Hydroxymethyl)benzyl)piperidin-4-ylamino)-4-nitrophenoxy)-3,5-dimethylbenzonitrile (5a4). Elution with petroleum ether/ethyl acetate/triethylamine (3:1:0.2). Orange solid, yield: 38.5%, mp: 163–165 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.25 (d, 1H, *J* = 7.2 Hz, NH), 8.13 (d, 1H, *J* = 9.4 Hz, C_6-Ph-H), 7.45 (s, 2H, C_3,C_5-Ph″-H), 7.34 (d, 4H, C_2,C_3,C_5,C_6-Ph′-H), 6.10(d, 1H, *J* = 2.5 Hz, C_3-Ph-H), 5.96 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 4.69 (s, 2H, Ph′-CH_2-O), 3.56 (s, 2H, Pi-CH_2-Ph′), 3.32 (m, 1H, C_1-Pi-H), 2.81 (t, 2H, Pi-CH_2), 2.23 (m, 2H, Pi-CH_2), 2.16 (s, 6H, 2× CH_3), 1.99 (t, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 162.81 (Ph-C_4), 153.79 (Ph″-C_1), 146.51, 140.08, 133.04, 132.99, 130.18, 129.38, 127.80, 127.03, 118.33 (C≡N), 109.81, 103.10, 97.68 (Ph-C_3), 65.06 (CH_2), 62.52 (CH_2), 51.24, 49.08, 31.36, 16.12 (2× CH_3). ESI-MS: *m*/*z* 487.5 (M+1), 509.4 (M+23). $C_{28}H_{30}N_4O_4$ [486.23].

4.1.5.5. 4-((4-(5-(4-Cyano-2,6-dimethylphenoxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzoic acid (5a5). Elution with ethyl acetate/methanol/acetic acid (12:1:1). Yellow solid, yield: 39.2%, mp: 230–232 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.29 (d, 1H, *J* = 6.2 Hz, NH), 8.14 (d, 1H, *J* = 9.4 Hz, C_6-Ph-H), 8.05 (d, 2H, *J* = 7.7 Hz, C_3,C_5-Ph′-H),7.47 (d, 2H, C_2,C_6-Ph′-H), 7.45 (s, 2H, C_3,C_5-Ph″-H), 6.13 (d, 1H, *J* = 1.3 Hz, C_3-Ph-H), 5.96 (dd, 1H, J_1 = 1.8 Hz, J_2 = 9.4 Hz, C_5-Ph-H), 3.77 (s, 2H, CH_2), 3.43 (m, 1H, C_1-Pi-H), 2.97 (t, 2H, Pi-CH_2), 2.49 (t, 2H, Pi-CH_2), 2.15 (s, 6H, 2× CH_3), 2.09 (m, 2H, Pi-CH_2), 1.79 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 162.87 (Ph-C_4), 153.72 (Ph″-C_1), 146.41, 133.03, 130.25, 130.16, 129.62, 127.83, 118.37 (C≡N), 109.82, 103.17, 97.70 (Ph-C_3), 62.00 (Pi-CH_2-Py), 53.44, 51.07, 30.56, 16.18 (2× CH_3). ESI-MS: *m*/*z* 499.5 (M−1). $C_{28}H_{28}N_4O_5$ [500.21].

4.1.5.6. 3,5-Dimethyl-4-(3-(1-(4-(methylsulfonyl)benzyl)piperidin-4-ylamino)-4-nitrophenoxy)benzonitrile (5a6). Elution with petroleum ether/ethyl acetate/triethylamine (5:1:0.1). Yellow solid, yield: 78.1%, mp: 164–166 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.28 (d, 1H, *J* = 7.0 Hz, NH), 8.14(d, 1H, *J* = 9.5 Hz, C_6-Ph-H), 7.91 (d, 2H, *J* = 8.2 Hz, C_3,C_5-Ph′-H), 7.58 (d, 2H, *J* = 7.2 Hz, C_2,C_6-Ph′-H), 7.46 (s, 2H, C_3,C_5-Ph″-H), 6.13 (d, 1H, *J* = 2.4 Hz, C_3-Ph-H), 5.97 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.64 (s, 2H, CH_2), 3.37 (m, 1H, C_1-Pi-H), 3.07 (s, 3H, SO_2–CH_3), 2.79 (t, 2H, Pi-CH_2), 2.26 (t, 2H, Pi-CH_2), 2.17 (s, 6H, 2× CH_3), 2.00 (m, 2H, Pi-CH_2), 1.70 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 162.85 (Ph-C_4), 153.75 (Ph″-C_1), 146.47, 133.04, 133.02, 130.24, 129.67, 127.77, 127.49, 118.39 (C≡N), 109.79, 103.15, 97.66 (Ph-C_3), 62.15 (Pi-CH_2-Py), 51.54, 44.53 (SO_2–CH_3), 31.42, 16.18 (2× CH_3). ESI-MS: *m*/*z* 535.3 (M+1), 557.2 (M+23). $C_{28}H_{30}N_4O_5S$ [534.19].

4.1.5.7. 4-((4-(5-(4-Cyano-2,6-dimethylphenoxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzamide (5a7). Elution with petroleum ether/ethyl acetate/triethylamine (2:1:0.15). Yellow solid, yield: 88.2%, mp: 198–200 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.26 (d, 1H, *J* = 7.1 Hz, NH), 8.14 (d, 1H, *J* = 9.4 Hz, C_6-Ph-H), 7.80 (d, 2H, *J* = 8.1 Hz, C_3,C_5-Ph′-H), 7.45 (s, 2H, C_3,C_5-Ph″-H), 7.43 (d, 2H, *J* = 8.3 Hz, C_2,C_6-Ph′-H), 6.10 (br, 2H, NH_2), 6.10 (d, 1H, *J* = 2.4 Hz, C_3-Ph-H), 5.98 (dd, 1H, J_1 = 2.4 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.61 (s, 2H, CH_2), 3.31 (m, 1H, C_1-Pi-H), 2.80 (t, 2H, Pi-CH_2), 2.24 (t, 2H, Pi-CH_2), 2.16 (s, 6H, 2× CH_3), 1.99 (m, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 169.22 (C=O), 162.83 (Ph-C_4), 153.77 (Ph″-C_1), 146.50, 133.05, 133.01, 132.33, 130.23, 129.20, 127.73, 127.49, 118.41 (C≡N), 109.75, 103.16, 97.61 (Ph-C_3), 62.37 (Pi-CH_2-Py), 51.39, 31.39, 16.17 (2× CH_3). ESI-MS: *m*/*z* 500.3 (M+1), 522.4 (M+23). $C_{28}H_{29}N_5O_4$ [499.22].

4.1.5.8. 4-((4-(5-(4-Cyano-2,6-dimethylphenoxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzenesulfonamide (5a8). Elution with petroleum ether/ethyl acetate/triethylamine (1:1:0.2). Yellow solid, yield: 65.6%, mp: 147–149 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.26 (d, 1H, *J* = 7.0 Hz, NH), 8.14 (d, 1H, *J* = 9.5 Hz, C_6-Ph-H), 7.90 (d, 2H, *J* = 8.0 Hz, C_3,C_5-Ph′-H), 7.50 (d, 2H, *J* = 8.0 Hz, C_2,C_6-Ph′-H), 7.46 (s, 2H, C_3,C_5-Ph″-H), 6.10 (d, 1H, C_3-Ph-H), 5.99 (dd, 1H, J_1 = 1.6 Hz, J_2 = 9.4 Hz, C_5-Ph-H), 5.20 (s, 2H, NH_2), 3.62 (s, 2H, CH_2), 3.35 (m, 1H, C_1-Pi-H), 2.78 (t, 2H, Pi-CH_2), 2.24 (t, 2H, Pi-CH_2), 2.16 (s, 6H, 2× CH_3), 1.99 (m, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 162.87 (Ph-C_4), 153.78 (Ph″-C_1), 146.51, 140.86, 133.05, 133.03, 130.23, 129.54, 127.70, 126.49, 118.43 (C≡N), 109.73, 103.25, 97.62 (Ph-C_3), 62.15 (Pi-CH_2-Py), 51.35, 31.37, 16.17 (2× CH_3). ESI-MS: *m*/*z* 536.4 (M+1), 558.4 (M+23). $C_{27}H_{29}N_5O_5S$ [535.19].

4.1.5.9. Methyl 4-((4-(5-(4-cyano-2,6-dimethylphenoxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzoate (5a9). Elution with petroleum ether/ethyl acetate (4:1). Yellow solid, yield: 71.4%, mp: 182–184 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.27 (d, 1H, *J* = 7.2 Hz, NH), 8.14 (d, 1H, *J* = 9.5 Hz, C_6-Ph-H), 8.00 (d, 2H, *J* = 8.3 Hz, C_3,C_5-Ph′-H),7.45 (s, 2H, C_3,C_5-Ph″-H), 7.42 (d, 2H, *J* = 8.1 Hz, C_2,C_6-Ph′-H), 6.13 (d, 1H, *J* = 2.5 Hz, C_3-Ph-H), 5.96 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.92 (s, 3H, COO–CH_3), 3.59 (s, 2H, CH_2), 3.34 (m, 1H, C_1-Pi-H), 2.79 (t, 2H, Pi-CH_2), 2.22 (t, 2H, Pi-CH_2), 2.16 (s, 6H, 2× CH_3), 1.99 (m, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 167.01 (C=O), 162.82 (Ph-C_4), 153.77 (Ph″-C_1), 146.53, 133.04, 133.01, 130.22, 129.66, 129.11, 128.84, 127.74, 118.40 (C≡N), 109.78, 103.04, 97.70 (Ph-C_3), 62.53 (Pi-CH_2-Py), 52.09 (O–CH_3), 51.54, 31.47, 16.18 (2× CH_3). ESI-MS: *m*/*z* 515.5 (M+1), 537.4 (M+23). $C_{29}H_{30}N_4O_5$ [514.22].

4.1.5.10. 4-(3-(1-Benzylpiperidin-4-ylamino)-4-nitrophenoxy)-3,5-dimethylbenzonitrile (5a10). Elution with petroleum ether/ethyl acetate (5:1). Yellow solid, yield: 72.0%, mp: 156–158 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.26 (d, 1H, *J* = 7.2 Hz, NH), 8.14 (d, 1H, *J* = 9.5 Hz, C_6-Ph-H), 7.45 (s, 2H, C_3,C_5-Ph″-H), 7.33 (m, 4H, C_2,C_3,C_5,C_6-Ph′-H), 7.26 (m, 1H, C_4-Ph′-H), 6.12 (d, 1H, *J* = 2.5 Hz, C_3-Ph-H), 5.95 (dd, 1H, J_1 = 2.5 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.53 (s, 2H, CH_2), 3.32 (m, 1H, C_1-Pi-H), 2.80 (t, 2H, Pi-CH_2), 2.20 (t, 2H, Pi-CH_2), 2.16 (s, 6H, 2× CH_3), 1.97 (m, 2H, Pi-CH_2), 1.65(m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ:

162.80 (Ph-C_4), 153.79 (Ph″-C_1), 146.59, 138.21, 133.05, 133.01, 130.21, 129.07, 128.30, 127.70, 127.14, 118.42 (C≡N), 109.77, 103.00, 97.70 (Ph-C_3), 63.00 (Pi-CH_2-Py), 51.50, 49.30, 31.59, 16.19 (2× CH_3). ESI-MS: *m*/*z* 457.5 (M+1). $C_{27}H_{28}N_4O_3$ [456.22].

4.1.5.11. *N*-(5-(Mesityloxy)-2-nitrophenyl)-1-(4-nitrobenzyl)piperidin-4-amine (5b1). Elution with petroleum ether/ethyl acetate (6:1). Orange solid, yield: 98.6%, mp: 178–180 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.28 (d, 1H, J = 7.2 Hz, NH), 8.19 (d, 2H, J = 8.6 Hz, C_3,C_5-Ph′-H), 8.11 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.52 (d, 2H, J = 8.6 Hz, C_2,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.15 (d, 1H, J = 2.4 Hz, C_3-Ph-H), 6.01 (dd, 1H, J_1 = 2.4 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.61 (s, 2H, CH_2), 3.38 (m, 1H, C_1-Pi-H), 2.76 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.25 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 2.00 (m, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 164.41 (Ph-C_4), 147.93, 147.20, 146.64, 146.49, 135.36, 130.47, 129.89, 129.70, 129.34, 127.21, 123.59, 103.75 (Ph-C_5), 97.53 (Ph-C_3), 62.12 (Pi-CH_2-Py), 51.61, 48.78, 31.48, 20.80 (Ph″-C_4-CH_3), 16.09 (2× CH_3). ESI-MS: *m*/*z* 491.4 (M+1), 513.4 (M+23). $C_{27}H_{30}N_4O_5$ [490.22].

4.1.5.12. *N*-(5-(Mesityloxy)-2-nitrophenyl)-1-(pyridin-4-ylmethyl)piperidin-4-amine (5b2). Elution with petroleum ether/ethyl acetate/triethylamine (1:1:0.1). Yellow solid, yield: 47.6%, mp: 105–107 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.56 (dd, 2H, J_1 = 1.4 Hz, J_2 = 4.6 Hz, C_3,C_5-Py-H), 8.28 (d, 1H, J = 7.0 Hz, NH), 8.11 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.30 (d, 2H, J = 5.5 Hz, C_2,C_6-Py-H), 6.91 (s, 2H, C_3,C_5-Ph′-H), 6.13 (d, 1H, J = 2.3 Hz, C_3-Ph-H), 6.02 (dd, 1H, J_1 = 2.4 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.55 (s, 2H, CH_2), 3.38 (m, 1H, C_1-Pi-H), 2.78 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph′-CH_3), 2.28 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph′-CH_3), 2.03 (m, 2H, Pi-CH_2), 1.69 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 164.42 (Ph-C_4), 149.88, 147.91, 146.62, 135.37, 130.45, 129.89, 129.70, 127.21, 123.80, 103.84 (Ph-C_5), 97.48 (Ph-C_3), 61.61 (Pi-CH_2-Py), 51.50, 31.27, 20.80 (Ph″-C_4-CH_3), 16.09 (2× CH_3). ESI-MS: *m*/*z* 447.5 (M+1), 469.5 (M+23). $C_{26}H_{30}N_4O_3$ [446.23].

4.1.5.13. 1-(4-((4-(5-(Mesityloxy)-2-nitrophenylamino)piperidin-1-yl)methyl)phenyl)ethanone (5b3). Elution with petroleum ether/ethyl acetate/triethylamine (5:1:0.1). Yellow solid, yield: 87.2%, mp: 138–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.27 (d, 1H, J = 6.9 Hz, NH), 8.10 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.93 (d, 2H, J = 8.1 Hz, C_3,C_5-Ph′-H), 7.44 (d, 2H, J = 7.8 Hz, C_2,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.13 (d, 1H, J = 1.9 Hz, C_3-Ph-H), 6.02 (dd, 1H, J_1 = 2.0 Hz, J_2 = 9.4 Hz, C_5-Ph-H), 3.59 (s, 2H, CH_2), 3.36 (m, 1H, C_1-Pi-H), 2.78 (t, 2H, Pi-CH_2), 2.60 (s, 3H, CO-CH_3), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.23 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 2.00 (m, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 197.80 (C═O), 164.40 (Ph-C_4), 147.94, 146.65, 136.26, 135.35, 130.46, 129.87, 129.69, 129.03, 128.45, 127.21, 103.79 (Ph-C_5), 97.50 (Ph-C_3), 62.50 (Pi-CH_2-Py), 51.52, 48.88, 31.58, 31.41, 26.62 (CO–CH_3), 20.79 (Ph″-C_4-CH_3), 16.07 (2× CH_3). ESI-MS: *m*/*z* 488.4 (M+1), 510.4 (M+23). $C_{29}H_{33}N_3O_4$ [487.25].

4.1.5.14. (4-((4-(5-(Mesityloxy)-2-nitrophenylamino)piperidin-1-yl)methyl)phenyl)methanol (5b4). Elution with petroleum ether/ethyl acetate/triethylamine (4:1:0.2). Yellow solid, yield: 62.7%, mp: 68–70 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.26 (d, 1H, J = 6.9 Hz, NH), 8.10 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.33 (d, 4H, C_2,C_3,C_5,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.12 (d, 1H, J = 1.9 Hz, C_3-Ph-H), 6.01 (dd, 1H, J_1 = 2.1 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 4.69 (s, 2H, Ph′-CH_2–O), 3.54 (s, 2H, Pi-CH_2-Ph′), 3.34 (m, 1H, C_1-Pi-H), 2.78 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.22 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 2.01 (m, 2H, Pi-CH_2), 1.66 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 164.40 (Ph-C_4), 147.93, 146.68, 139.99, 135.36, 130.47, 129.87, 129.70, 129.41, 127.16, 127.06, 103.79 (Ph-C_5), 97.50 (Ph-C_3), 65.12 (CH_2), 62.61 (CH_2), 51.34, 31.60, 31.29, 20.80 (Ph″-C_4-CH_3), 16.09 (2× CH_3). ESI-MS: *m*/*z* 476.3 (M+1), 498.4 (M+23). $C_{28}H_{33}N_3O_4$ [475.25].

4.1.5.15. 4-((4-(5-(Mesityloxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzoic acid (5b5). Yellow solid, yield: 78.3%, mp: 133–135 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.28 (d, 1H, J = 6.2 Hz, NH), 8.10 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 8.04 (d, 2H, J = 7.5 Hz, C_3,C_5-Ph′-H), 7.44 (d, 2H, J = 7.7 Hz, C_2,C_6-Ph′-H), 6.90 (s, 2H, C_3,C_5-Ph″-H), 6.11 (d, 1H, C_3-Ph-H), 6.03 (dd, 1H, J_1 = 1.4 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.84 (s, 2H, CH_2), 3.44 (m, 1H, C_1-Pi-H), 3.01 (t, 2H, Pi-CH_2), 2.56 (t, 2H, Pi-CH_2), 2.30 (s, 3H, C_4-Ph″-CH_3), 2.06 (m, 2H, Pi-CH_2), 2.06 (s, 6H, C_2,C_6-Ph″-CH_3), 1.80 ((m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 167.97 (COOH), 164.30 (Ph-C_4), 147.91, 146.72, 143.62, 135.44, 131.98, 131.00, 130.39, 130.09, 129.68, 129.07, 128.92, 126.91, 103.96 (Ph-C_5), 98.26 (Ph-C_3), 62.02 (Pi-CH_2-Py), 51.50, 31.43, 30.47, 20.81 (Ph″-C_4-CH_3), 16.12 (2× CH_3). ESI-MS: *m*/*z* 490.4 (M+1), 512.5 (M+23). $C_{28}H_{31}N_3O_5$ [489.23].

4.1.5.16. *N*-(5-(Mesityloxy)-2-nitrophenyl)-1-(4-(methylsulfonyl)benzyl)piperidin-4-amine (5b6). Elution with petroleum ether/ethyl acetate/triethylamine (5:1:0.1). Yellow solid, yield: 74.0%, mp: 136–138 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.28 (d, 1H, J = 7.0 Hz, NH), 8.10 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.90 (d, 2H, J = 8.2 Hz, C_3,C_5-Ph′-H), 7.56 (d, 2H, J = 7.9 Hz, C_2,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.14 (d, 1H, J = 1.9 Hz, C_3-Ph-H), 6.01 (dd, 1H, J_1 = 2.0 Hz, J_2 = 9.4 Hz, C_5-Ph-H), 3.61 (s, 2H, CH_2), 3.38 (m, 1H, C_1-Pi-H), 3.07 (s, 3H,SO_2-CH_3), 2.77 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.24 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 2.00 (m, 2H, Pi-CH_2), 1.67 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 164.42 (Ph-C_4), 147.91, 146.64, 139.32, 135.37, 130.46, 129.90, 129.71, 129.63, 127.46, 127.19, 103.78 (Ph-C_5), 97.51 (Ph-C_3), 62.23 (Pi-CH_2-Py), 51.52, 44.55 (SO_2–CH_3), 31.42, 20.82 (Ph″-C_4-CH_3), 16.10 (2× CH_3). ESI-MS: *m*/*z* 524.5(M+1), 541.5 (M+18), 546.4 (M+23). $C_{28}H_{33}N_3O_5S$ [523.21].

4.1.5.17. 4-((4-(5-(Mesityloxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzamide (5b7). Elution with petroleum ether/ethyl acetate/triethylamine (3:1:0.27). Yellow solid, yield: 92.8%, mp: 92–94 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.27 (d, 1H, J = 6.8 Hz, NH), 8.10 (d, 1H, J = 9.5 Hz, C_6-Ph-H), 7.79 (d, 2H, J = 8.0 Hz, C_3,C_5-Ph′-H), 7.43 (d, 2H, J = 7.9 Hz, C_2,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.13 (d, 1H, C_3-Ph-H), 6.06 (br, 2H, NH_2), 6.01 (dd, 1H, J_1 = 1.6 Hz, J_2 = 9.4 Hz, C_5-Ph-H), 3.58 (s, 2H, CH_2), 3.36 (m, 1H, C_1-Pi-H), 2.77 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.23 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 2.01 (m, 2H, Pi-CH_2), 1.66 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 169.25 (C═O), 164.42 (Ph-C_4), 147.91, 146.68, 135.36, 132.25, 130.46, 129.88, 129.70, 129.13, 127.46, 127.15, 103.78 (Ph-C_5), 97.51 (Ph-C_3), 62.47 (Pi-CH_2-Py), 51.49, 31.60, 31.40, 20.81 (Ph″-C_4-CH_3), 16.10 (2× CH_3). ESI-MS: *m*/*z* 489.6 (M+1), 511.5 (M+23). $C_{28}H_{32}N_4O_4$ [488.24].

4.1.5.18. 4-((4-(5-(Mesityloxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzenesulfonamide (5b8). Elution with petroleum ether/ethyl acetate/triethylamine (1:1:0.2). Yellow solid, yield: 75.3%, mp: 128–130 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.27 (d, 1H, J = 6.4 Hz, NH), 8.10 (d, 1H, J = 9.4 Hz, C_6-Ph-H), 7.89 (d, 2H, J = 7.8 Hz, C_3,C_5-Ph′-H), 7.49 (d, 2H, J = 7.8 Hz, C_2,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.14 (d, 1H, C_3-Ph-H), 6.01 (dd, 1H, J = 9.2 Hz, C_5-Ph-H), 5.14 (s, 2H, NH_2), 3.59(s, 2H, CH_2), 3.37 (m, 1H, C_1-Pi-H), 2.74 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.24 (t,

2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 1.98 (m, 2H, Pi-CH_2), 1.66 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 164.45 (Ph-C_4), 147.90, 146.66, 140.83, 135.38, 130.45, 129.90, 129.71, 129.55, 127.16, 126.51, 103.84 (Ph-C_5), 97.52 (Ph-C_3), 62.18 (Pi-CH_2-Py), 60.44, 51.31, 31.27, 20.81 (Ph″-C_4-CH_3), 16.10 (2× CH_3). ESI-MS: *m*/*z* 525.6(M+1), 547.2 (M+23). $C_{27}H_{32}N_4O_5S$ [524.21].

4.1.5.19. Methyl 4-((4-(5-(mesityloxy)-2-nitrophenylamino)piperidin-1-yl)methyl)benzoate (5b9). Elution with petroleum ether/ethyl acetate (7:1). Yellow solid, yield: 81.5%, mp: 168–170 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.27 (d, 1H, *J* = 7.0 Hz, NH), 8.10 (d, 1H, *J* = 9.5 Hz, C_6-Ph-H), 8.00 (d, 2H, *J* = 8.2 Hz, C_3,C_5-Ph′-H), 7.42 (d, 2H, *J* = 7.9 Hz, C_2,C_6-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.13 (d, 1H, *J* = 2.1 Hz, C_3-Ph-H), 6.01 (dd, 1H, J_1 = 2.2 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.92 (s, 3H, COO–CH_3), 3.58 (s, 2H, CH_2), 3.35 (m, 1H, C_1-Pi-H), 2.77 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.23 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 2.01 (m, 2H, Pi-CH_2), 1.66(m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 167.02 (C═O), 164.40 (Ph-C_4), 147.92, 146.67, 135.36, 130.47, 129.89, 129.70, 129.67, 129.09, 128.88, 127.16, 103.77 (Ph-C_5), 97.50 (Ph-C_3), 62.56 (Pi-CH_2-Py), 52.10 (O–CH_3), 51.57, 31.42, 20.82 (Ph″-C_4-CH_3), 16.10 (2× CH_3). ESI-MS: *m*/*z* 504.4 (M+1), 526.4 (M+23). $C_{29}H_{33}N_3O_5$ [503.24].

4.1.5.20. 1-Benzyl-*N*-(5-(mesityloxy)-2-nitrophenyl)piperidin-4-amine (5b10). Elution with petroleum ether/ethyl acetate (16:1). Yellow solid, yield: 84.0%, mp: 95–97 °C. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 8.27 (d, 1H, *J* = 7.0 Hz, NH), 8.10 (d, 1H, *J* = 9.5 Hz, C_6-Ph-H), 7.32 (m, 4H, C_2,C_3,C_5,C_6-Ph′-H), 7.25 (m, 1H, C_4-Ph′-H), 6.91 (s, 2H, C_3,C_5-Ph″-H), 6.12 (d, 1H, *J* = 2.4 Hz, C_3-Ph-H), 6.01 (dd, 1H, J_1 = 2.4 Hz, J_2 = 9.5 Hz, C_5-Ph-H), 3.53 (s, 2H, CH_2), 3.33 (m, 1H, C_1-Pi-H), 2.79 (t, 2H, Pi-CH_2), 2.31 (s, 3H, C_4-Ph″-CH_3), 2.19 (t, 2H, Pi-CH_2), 2.07 (s, 6H, C_2,C_6-Ph″-CH_3), 1.98 (m, 2H, Pi-CH_2), 1.64 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ: 164.38 (Ph-C_4), 147.95, 146.73, 138.21, 135.35, 130.49, 129.87, 129.70, 129.10, 128.30, 127.16, 127.14, 103.74 (Ph-C_5), 97.51 (Ph-C_3), 63.01 (Pi-CH_2-Py), 51.50, 49.32, 31.51, 20.82 (Ph″-C_4-CH_3), 16.11 (2× CH_3). ESI-MS: *m*/*z* 446.5 (M+1). $C_{27}H_{31}N_3O_3$ [445.24].

4.1.6. General procedure for the synthesis of 4-((4-(2-amino-5-(4-cyano-2,6-dimethylphenoxy)phenylamino)piperidin-1-yl)methyl)benzenesulfonamide hydrochloride (7a1)

To palladium–carbon catalyst (0.014 g, 10%) was added absolute ethyl alcohol (1 ml) gently, then the suspension was added to **5a8** (0.07 g) in THF (0.5 ml). The reaction mixture was exposed to hydrogen at 30 °C and stirred for 36 h. The suspension was filtered and the filtrate was purified to give **6a1** as slight pink syrup. Subsequently, **6a1** was redissolved in DCM (0.5 ml) immediately and concentrated hydrochloric acid (4 drops) was added to this solution in an ice-bath. The mixture continued stirring for 0.5 h, then the solvent was removed under reduced pressure and the residue was recrystallized from ethanol to give the compound **7a1** as a white solid. Yield: 56.3%, mp: 207–209 °C. ^{1}H NMR (400 MHz, D_2O, ppm) δ: 7.94 (d, 2H, *J* = 8.0 Hz, C_3,C_5-Ph′-H), 7.66 (d, 2H, *J* = 8.1 Hz, C_2,C_6-Ph′-H), 7.47 (s, 2H, C_3,C_5-Ph″-H), 7.09 (d, 1H, *J* = 8.7 Hz, C_6-Ph-H), 6.40 (d, 1H, *J* = 2.1 Hz, C_3-Ph-H), 6.01 (d, 1H, *J* = 8.6 Hz, C_5-Ph-H), 4.37 (s, 2H, CH_2), 3.53 (t, 2H, Pi-CH_2), 3.31 (m, 1H, C_1-Pi-H), 3.08 (m, 2H, Pi-CH_2), 2.21 (t, 2H, Pi-CH_2), 2.01 (s, 6H, 2× CH_3), 1.65 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, D_2O, ppm) δ: 158.15 (Ph″-C_1), 154.62 (Ph-C_4), 143.01 (Ph′-C_4), 141.70 (Ph′-C_1), 133.81, 133.63, 133.52, 133.33, 132.38, 132.20, 126.78, 125.75, 119.72 (C≡N), 111.65, 108.08, 103.92, 101.17, 59.52 (Pi-CH_2-Py), 51.69, 47.50, 28.89, 15.34 (2× CH_3). ESI-MS: *m*/*z* 506.4 (M+1), 528.4 (M+23). $C_{27}H_{31}N_5O_3S$ [505.21].

4.1.7. General procedure for the synthesis of *tert*-butyl 4-(2-acetamido-5-(4-cyano-2,6-dimethylphenoxy)phenylamino)piperidine-1-carboxylate (9)

To palladium–carbon catalyst (0.05 g, 10%) was added absolute ethyl alcohol (8 ml) gently, then the suspension was added to **3a** (0.5 g, 1.07 mmol) in THF (2 ml). The reaction mixture was exposed to hydrogen at 30 °C and stirred for 6 h. The suspension was filtered, the filtrate was concentrated and the solvent was replaced with dichloromethane. Then triethylamine (0.16 g, 1.6 mmol) and acetylchloride (0.1 g, 1.28 mmol) was added successively in an ice-bath and stirred for 1 h. The reaction mixture was washed with water for 3 times, and then the water layer was extracted with dichloromethane for 3 times. The combined organic layers were dried over anhydrous sodium sulfate, purified by column chromatography on silica and recrystallized from diethyl ether to afford the intermediate **9** as a creamy white solid. Yield: 80.39%. ESI-MS: *m*/*z* 479.4 (M+1), 501.4 (M+23). $C_{27}H_{34}N_4O_4$ [478.26].

4.1.8. General procedure for the synthesis of *N*-(4-(4-cyano-2,6-dimethylphenoxy)-2-(piperidin-4-ylamino)phenyl)acetamide (10)

Follow the procedure in the preparation of **4a** and **4b** to give the compound **10** as a white solid. Yield: 96.9%.

4.1.9. General procedure for the synthesis of *N*-(4-(4-cyano-2,6-dimethylphenoxy)-2-(1-(4-sulfamoylbenzyl)piperidin-4-ylamino)phenyl)acetamide (11)

Follow the procedure in the preparation of **5a** and **5b** using EtOAc/methanol/triethylamine (10/1/0.2) as eluant to afford the product as colorless syrup. Yield: 62.2%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.00(s, 1H, Ph-NH-CO), 7.80 (d, 2H, *J* = 8.2 Hz, C_3,C_5-Ph′-H), 7.67 (s, 2H, C_3,C_5-Ph″-H), 7.50 (d, 2H, *J* = 8.2 Hz, C_2,C_6-Ph′-H), 7.32 (s, 2H, NH_2), 6.97 (d, 1H, *J* = 8.6 Hz, C_6-Ph-H), 6.20 (d, 1H, *J* = 2.3 Hz, C_3-Ph-H), 5.71 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.5 Hz, C_5-Ph-H), 4.80(d, 1H, *J* = 7.5 Hz, Pi-NH), 3.55 (s, 2H, CH_2), 3.41 (t, 2H, Pi-CH_2), 3.17 (m, 1H, C_1-Pi-H), 2.75 (t, 2H, Pi-CH_2), 2.11 (s, 6H, 2× CH_3), 2.07 (s, 3H, CO–CH_3), 1.87 (m, 2H, Pi-CH_2), 1.44 (m, 2H, Pi-CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 169.21 (C═O), 155.81, 155.24, 143.66, 143.17, 133.52, 133.36, 129.38, 127.97, 126.08, 119.14, 118.89 (C≡N), 108.18, 100.59, 98.63 (Ph-C_3), 61.85 (Pi-CH_2-Py), 52.22, 49.44, 31.97, 23.67 (CO–**C**H_3), 16.14 (2× CH_3). ESI-MS: *m*/*z* 548.5 (M+1). $C_{29}H_{33}N_5O_4S$ [547.23].

4.2. *In vitro* anti-HIV assay

Evaluation of the antiviral activity of the compounds was performed using the MTT assay as previously described.[23,27] Stock solutions (10× final concentration) of test compounds were added in 25 µl volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock-and HIV-infected cells at the beginning of each experiment. Serial fivefold dilutions of the test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV-and mock-infected cell samples were included for each sample. HIV-1 (III_B), HIV-1 (RES056) or HIV-2 (ROD)[28,29] stock (50 µl) at 100–300 $CCID_{50}$ (50% cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compounds on uninfected cells in order to assess its cytotoxicity. Exponentially growing MT-4 cells[30] were centrifuged for 5 min at 1000 rpm and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/ml, and 50 µl volumes were transferred to the microtiter tray wells. Five days after infection, the via-

bility of mock-and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay was based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of two or three wells.

The 50% effective antiviral concentration (EC_{50}) was defined as the concentration of the tested compound achieving 50% protection from viral cytopathicity. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration that reduced the viability of mock-infected cells by 50%. The symbol '>' was used to indicate the highest concentration at which the compound was tested and still found to be non-cytotoxic.

4.3. HIV-1 RT inhibition assay

The inhibition assay of HIV-1 RT_{wt} was implemented by utilizing the template/primer hybrid poly(A) × oligo$(dT)_{15}$, digoxigenin-and biotin-labeled nucleotides, an antibody to digoxigenin which conjugated to peroxidase (anti-DIG-POD), and the peroxidase substrate ABTS.[31] The incorporation quantities of the digoxigenin- and biotin-labeled dUTP into DNA represented the activity of HIV-1 RT. The IC_{50} value corresponded to the concentration of the tested compound required to inhibit the incorporation of the labeled dUTP by 50%.

Acknowledgments

The financial support from the National Natural Science Foundation of China (NSFC Nos. 81273354, 81102320, 30873133, 30772629, 30371686), Key Project of NSFC for International Cooperation (No. 30910103908), Research Fund for the Doctoral Program of Higher Education of China (Nos. 20110131130005, 20110131120037 and No. 070422083), Key Project of The International Cooperation, Ministry of Science and Technology of China (2003DF000033) and KU Leuven (GOA 10/014) is gratefully acknowledged. We thank K. Erven, K. Uyttersprot and C. Heens for technical assistance with the HIV assays.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.033. These data include MOL files and InChiKeys of the most important compounds described in this article.

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