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Discovery of Piperidine-Linked Pyridine Analogues as Potent Non-nucleoside HIV-1 Reverse Transcriptase Inhibitors

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In our continued efforts to discover more active and less toxic HIV-1 non-nucleoside reverse transcriptase inhibitors, we recently designed a novel series of piperidine-linked pyridine analogues on the basis of diarylpyrimidine derivatives, among which two drugs—etravirine and rilpivirine—are approved for use by the US FDA. The title compounds were evaluated for activity against wild-type and resistant mutant strains of HIV-1 as well as HIV-2 in MT-4 cells. The highly potent compound **BD-c1** ($EC_{50}=10$ nM, $CC_{50}\geq 146$ μM, $SI\geq 14126$) displays lower cytotoxicity and higher selectivity than etravirine ($EC_{50}=2.2$ nM, $CC_{50}=28$ μM, $SI=12884$) against wild-type HIV-1. Compound **BD-e2** ($EC_{50}=5.1$ nM) shows greater antiviral efficacy against wild-type HIV-1 than do the four reference drugs nevirapine, delavirdine, efavirenz, and zidovudine. Many compounds were also found to be active against the frequently observed drug-resistant double mutant (K103N+Y181C) HIV-1 strain. Herein we report the design, synthesis, anti-HIV evaluation, preliminary structure–activity relationships, and molecular simulations of novel piperidine-linked pyridine analogues.

Introduction

According to statistics reported by the World Health Organization (WHO)/UNAIDS regarding the acquired immunodeficiency syndrome (AIDS) epidemic, there were 34 million people infected with human immunodeficiency virus (HIV) in 2012,[1] underscoring the importance of continued drug discovery efforts against this virus. Although unprecedented success has been realized by several drugs approved for the treatment of HIV infections[2] and use of the highly active antiretroviral therapy (HAART) regimen, undesirable toxic side effects along with rapid emergence of drug resistance resulting from prolonged HAART often compromise clinical application and effectiveness of treatment.[3] Therefore, the discovery and development of novel anti-HIV drugs with high potency and low toxicity is clearly needed.

HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs), with five drugs approved by the US Food and Drug Administration (FDA), have become an indispensable component of first-line drug regimens. Both etravirine and rilpivirine are structural members of the diarylpyrimidine (DAPY) family of derivatives, which have attracted considerable attention in diverse structural modifications and optimizations over the past decade.[4] Among the structural modifications, DAPY derivatives with piperidine substitutions at the right wing (compound **1**) show excellent activity against HIV-1 replication (wild-type $EC_{50}=0.3$ nM).[5] Encouraged by this result, we designed and synthesized piperidine-linked triazine derivatives that show high potency and low cytotoxicity (compound **2**, wild-type $EC_{50}=4.61$ nM, $SI=5945$, Figure 1).[6] In our continued efforts to discover more active and less toxic HIV-1 NNRTIs, we recently designed a series of piperidine-linked pyridine analogues (Figure 2) with the following structural

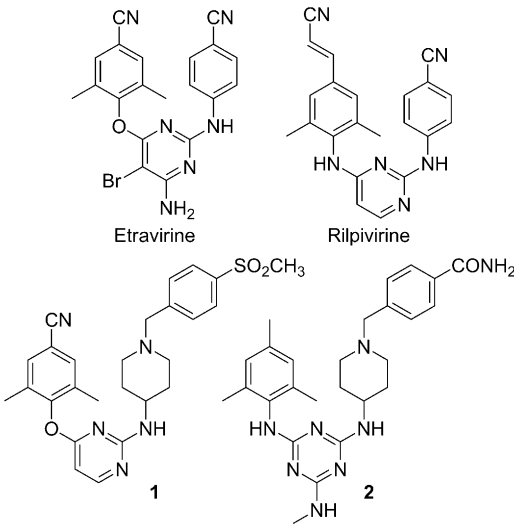

Figure 1. Structures of HIV-1 NNRTIs.

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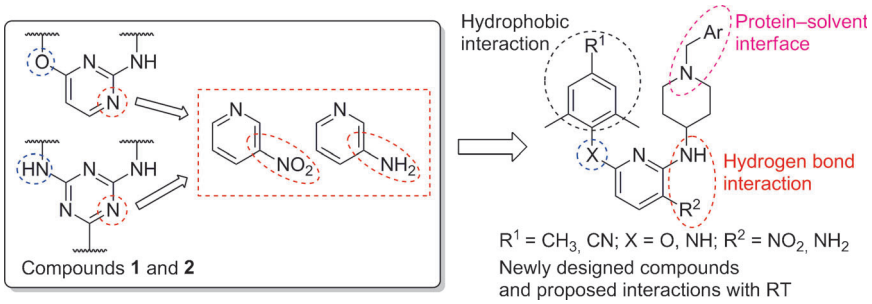


Figure 2. Design strategies of new piperidine-linked pyridine compounds.

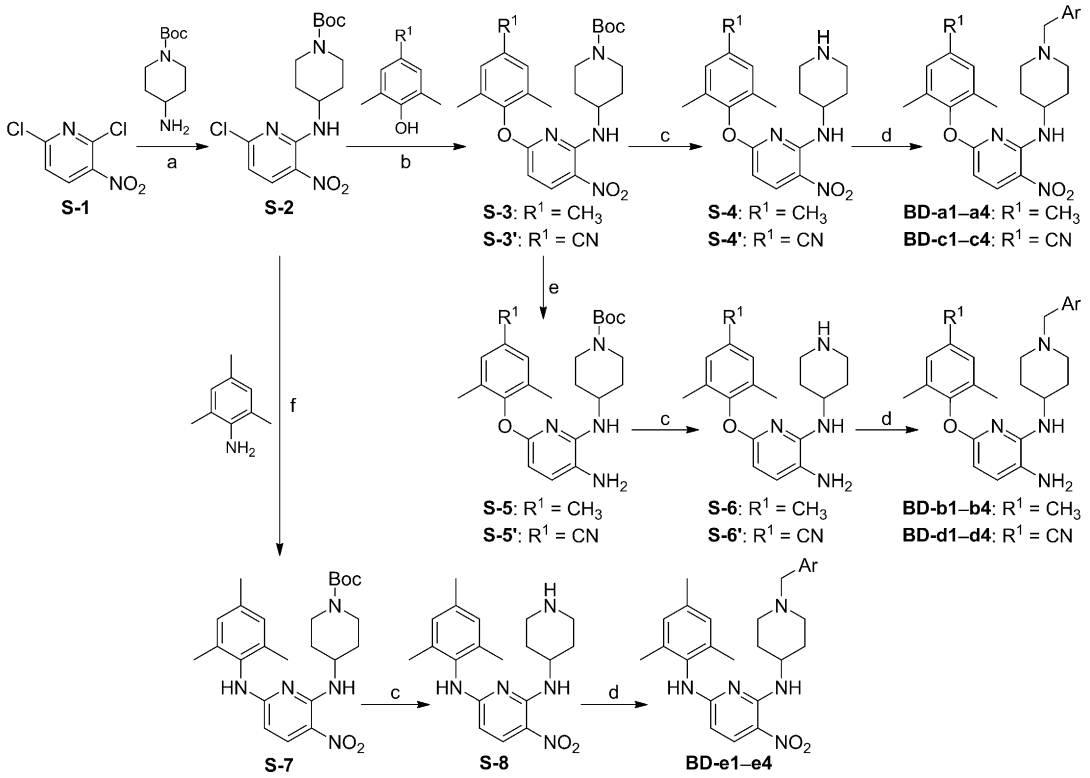
characteristics: 1) a nitro or amino group was introduced onto pyridine ring as a potential hydrogen bond acceptor or donor instead of the N atom of the pyrimidine or triazine ring, which was observed similarly in diarylpyridine NNRTIs;[7] 2) the *para* substituents (R^1 = Me, CN) of 2,4,6-trisubstituted phenol or aniline, and the linker (X = O, NH) were taken into consideration for combining the lead compounds **1** and **2**. Also, the heterocyclic aryl (Ar) group, and phenyl group with polar hydrophilic substituents, were designed to orient directly into the protein–solvent interface, as reported previously.

Results and Discussion

Chemistry

An expeditious synthesis of the novel piperidine-linked pyridine analogues was carried out in our research group. As depicted in Scheme 1, the intermediate **S-2** was readily obtained through nucleophilic substitution between 4-amino-1-Boc-piperidine and the starting material 2,6-dichloro-3-nitropyridine (**S-1**) at room temperature with good yield (85%).[8] Coupling intermediate **S-2** and sterically hindered 2,4,6-trimethylphenol (3 equiv) with cesium carbonate (4.5 equiv) as the base in dioxane at reflux for 7 h gave the key intermediate **S-3**. The reduced product **S-5** was prepared via catalytic hydrogenation of intermediate **S-3** under Pd-C/H_2/MeOH conditions at room temperature in satisfactory yield (98%). Accordingly, intermediates **S-3′** and **S-5′** with R^1 = CN were obtained by following the same procedure as the preparation of **S-3** and **S-5**. The mixture of **S-2** and the liquid 2,4,6-trimethylaniline (2 equiv) was heated at 130 °C without the addition of solvent for 5 h to afford key intermediate **S-7**.

Intermediates **S-3**, **S-3′**, **S-5**, **S-5′**, and **S-7** were dissolved in dichloromethane in the presence of trifluoroacetic acid (TFA, 10 equiv) and stirred at room temperature to yield intermedi-



Scheme 1. Synthesis of compounds **BD-a1–a4**, **BD-b1–b4**, **BD-c1–c4**, **BD-d1–d4** and **BD-e1–e4**. *Reagents and conditions*: a) Na_2CO_3, EtOH, RT, overnight; b) Cs_2CO_3, dioxane, reflux, 7 h; c) TFA, CH_2Cl_2, RT, overnight; d) substituted benzyl chloride or 4-picolyl chloride hydrochloride, K_2CO_3, acetone, RT, overnight; e) Pd-C, H_2, MeOH, RT, overnight; f) Δ (130 °C), 5 h.

ates **S-4**, **S-4′**, **S-6**, **S-6′**, and **S-8**, respectively, with yields >90%.[9] These were alkylated at the N atom of piperidine to afford the title compounds **BD-a1**–**a4**, **BD-b1**–**b4**, **BD-c1**–**c4**, **BD-d1**–**d4**, and **BD-e1**–**e4**.

Melting point, thin-layer chromatography (TLC), ^{1}H NMR, and ESIMS analysis of all final compounds, as well as HRMS data and ^{13}C NMR spectra for representative compounds from each sub-series, were determined and are in full agreement with the proposed structures.

Table 1. Anti-HIV activity and cytotoxicity in MT-4 cells infected with wild-type HIV-1 (III_B) and HIV-2 (ROD).

Compd	Ar	EC_{50}[a]		CC_{50} [μM][b]	SI[c]	
		HIV-1 [nM]	HIV-2 [μM]		HIV-1	HIV-2
BD-a1	4-SO_2Me-Ph	18±12	>24	24±1.8	1375	<1
BD-a2	4-SO_2NH_2-Ph	8.5±1.6	>22	22±1.9	2600	<1
BD-a3	4-$CONH_2$-Ph	12±5.3	>19	19±7.9	1600	<1
BD-**a4**	pyridin-4-yl	833±651	>279	>279	>335	–
BD-b1	4-SO_2Me-Ph	116±55	>28	28±1.5	241	<1
BD-b2	4-SO_2NH_2-Ph	112±97	>26	26±2.9	234	<1
BD-b3	4-$CONH_2$-Ph	58±5.7	>26	26±4.0	459	<1
BD-b4	pyridin-4-yl	84±9.2	>30	30±3.5	358	<1
BD-c1	4-SO_2Me-Ph	10±2.5	≥146	≥146	≥14126	–
BD-c2	4-SO_2NH_2-Ph	19±6.9	>27	27±1.6	1470	<1
BD-c3	4-$CONH_2$-Ph	31±7.0	>27	27±1.1	877	<1
BD-c4	pyridin-4-yl	39±10	≥6.7	26±3.4	654	≤4
BD-d1	4-SO_2Me-Ph	47±15	>28	28±3.0	607	<1
BD-d2	4-SO_2NH_2-Ph	53±23	>24	24±1.0	456	<1
BD-d3	4-$CONH_2$-Ph	36±13	>27	27±2.5	742	<1
BD-d4	pyridin-4-yl	56±11	>31	31±1.1	559	<1
BD-e1	4-SO_2Me-Ph	7.8±0.77	>21	21±9.1	2662	<1
BD-e2	4-SO_2NH_2-Ph	5.1±2.4	>14	14±8.0	2692	<1
BD-e3	4-$CONH_2$-Ph	9.6±1.5	>25	25±2.1	2645	<1
BD-e4	pyridin-4-yl	10±2.6	>31	31±5.8	2935	<1
1[5]		0.3				
2[6]		4.61±1.90	>27.39	27.39±2.34	5945	<1
NVP		113±69	ND[d]	>15	>132	ND
DLV		107±69	ND	>36	>338	ND
EFV		8.7±1.5	ND	>6.3	>727	ND
AZT		6.5±2.4	0.006±0.001	>93	>14445	>15690
ETV[f,11e]		2.2±0.4	>28	28±12	12884	<1

[a] Compound concentration required for 50% protection of MT-4 cells against HIV-1-induced cytotoxicity; data represent the mean ±SD of at least two separate experiments performed in triplicate. [b] Compound concentration required to decrease normal uninfected cell viability by 50%.; data represent the mean ±SD of at least two separate experiments performed in triplicate. [c] Selectivity index: CC_{50}/EC_{50}; "–" indicate incalculable values. [d] Not determined. [e] Data were obtained from the same research group using the same method.

Anti-HIV activity in MT-4 cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as reported previously,[6,10] was used to evaluate the newly synthesized compounds along with four reference drugs: nevirapine (NVP), delavirdine mesylate (DLV), efavirenz (EFV), and zidovudine (AZT). These compounds were assayed for their anti-HIV activity and cytotoxicity in MT-4 cells infected with wild-type HIV-1 (III_B), resistant mutant strain (K103N+Y181C) of HIV-1, as well as HIV-2 (ROD strain). Results of the biological evaluations, expressed as EC_{50} (50% HIV-1 replication inhibitory concentration), CC_{50} (50% cytotoxic concentration), and SI (selectivity index, given by the CC_{50}/EC_{50} ratio) values, are summarized in Tables 1 and 2, and data represent the mean of at least two separate experiments.

All the newly designed and synthesized compounds were active against wild-type HIV-1, with EC_{50} values ranging from 5.1 nM to 832 nM, and most compounds exhibited stronger inhibitory activity than those of NVP and DLV, as listed in Table 1. Compound **BD-e2** was the most potent compound, with an EC_{50} value of 5.1 nM, which proved lower than those of the four reference drugs NVP, DLV, EFV, and AZT, but higher than that of the newly FDA-approved drug etravirine (ETV) against wild-type HIV-1 (EC_{50}=2.2 nM,[11] assayed in the same research group using the same method). Compound **BD-c1** (EC_{50}=10 nM) showed significantly low cytotoxicity, with a CC_{50} value >146 μM and high selectivity, with an SI value >14126, making **BD-c1** a promising lead compound similar to the new drug ETV (CC_{50}=28 μM, SI=12884). Furthermore, none of the newly synthesized compounds were active against HIV-2 (ROD) at a subtoxic concentration in MT-4 cells.

Notably, most of the compounds (**BD-a3**, **BD-b1**–**b4**, **BD-c4**, and **BD-d1**–**d4**) displayed low-micromolar activities against a drug-resistant mutant strain of HIV-1 (K103N+Y181C), which is a frequently encountered mutant resulting from the treatment of AIDS patients with HIV-1 NNRTIs.[12] As the data in Table 2 indicate, the compounds show activity (EC_{50} values) in the low-micromolar concentration range and good relative activity (fold resistance). Compounds **BD-d1** (EC_{50}=0.99 μM) and **BD-d2** (EC_{50}=0.99 μM) show better activities than the reference drugs NVP and DLV, and the relative activity of the two compounds (respective fold resistance: 21 and 19) is superior to that of the reference drugs NVP, DLV, and EFV, but still inferior to that of the new drug ETV (fold resistance: 16).

Preliminary structure–activity relationship (SAR) information based on the results of the antiviral assays indicates the following: 1) by comparison with the activities between compounds **BD-a1**–**a4** and **BD-e1**–**e4**, compounds with an –NH– linker are more active than those with an –O– linker; 2) reduction of the $-NO_2$ group (compounds **BD-a1**–**a3** and **BD-c1**–**c4**) to an $-NH_2$ group (compounds **BD-b1**–**b3** and **BD-d1**–**d4**) decreased the activity against wild-type HIV-1, but increased the activity against the resistant mutant strain of HIV-1; 3) compounds with a 4-cyano-2,6-dimethylphenyl group (R^1=CN, **BD-d1**–**d4**), with the same moiety as the new drug ETV, showed better activity against wild-type and the resistant mutant strain of HIV-1 than compounds with a 2,4,6-trimethylphenyl group (R^1=

Table 2. Anti-HIV activities and cytotoxicity in MT-4 cells infected with resistant mutant strain (K103N + Y181C).

Compd	EC_{50} [μM]	FR[a]	CC_{50} [μM]	SI
BD-a1	>24	>1374	24±1.8	<1
BD-a2	≥5.7	≥673	22±1.9	≤4
BD-a3	5.8±0.9	500	19±7.8	3
BD-a4	≥239	≥287	>279	–
BD-b1	5.6±2.5	48	28±1.5	5
BD-b2	1.5±0.58	14	26±2.9	17
BD-b3	3.9±0.58	68	26±4.0	7
BD-b4	2.5±1.3	30	30±3.5	12
BD-c1	≥32	≥3045	≥146	–
BD-c2	≥7.6	≥410	27±1.6	≤4
BD-c3	>27	>876	27±1.1	<1
BD-c4	7.7±3.5	198	25±3.3	3
BD-d1	0.99±0.06	21	28±3.0	29
BD-d2	0.99±0.01	19	24±1.0	24
BD-d3	1.1±0.08	31	27±2.5	24
BD-d4	1.0±0.2	18	31±1.1	30
BD-e1	>21	>2662	21±9.1	<1
BD-e2	≥5.9	≥1156	14±8.0	≤2
BD-e3	>25	>2645	25±2.1	<1
BD-e4	>31	>2934	31±5.8	<1
1[5]	0.020	67		
2[6]	0.56±0.12	121.6	27.39±2.34	49
NVP	5.1±5.1	45	>15	>3
DLV	>36	>338	>36	–
EFV	0.5±0.1	58	>6.3	>12
AZT	0.0063±0.00003	1.0	>93	≥14445
ETV[, 11b]	0.034±0.005	15	28±11	817

[a] Fold resistance: ratio of EC_{50} value against the drug-resistant strain over EC_{50} value against wild-type HIV-1 ($EC_{50}^{mutant}/EC_{50}^{wt}$); data represent the mean ±SD of at least two separate experiments performed in triplicate. [b] Data were obtained from the same research group using the same method.

Me, **BD-b1**–**b4**). The biological evaluation results and the important SAR information analyzed above will be beneficial in the future design of new potent compounds.

Inhibition of HIV-1 RT

To directly prove that the newly synthesized compounds target HIV-1 RT, the active compound **BD-c1** was selected to carry out recombinant HIV-1 RT inhibitory assays using the template/primer hybrid poly(A)·oligo$(dT)_{15}$ (RT kit, Roche). The results show that **BD-c1** displays good, but slightly inferior inhibitory activity (IC_{50} = 1.2 μM) against HIV-1 RT relative to the reference drug ETV (IC_{50} = 0.94 μM) (Table 3). Thus, the newly synthesized piperidine-linked pyridine analogues do indeed inhibit the activity of HIV-1 RT effectively, and therefore can be classified as HIV-1 RT inhibitors.

Table 3. In vitro recombinant HIV-1 RT inhibitory assay of compounds **BD-c1** and ETV.

Compd	IC_{50} [μM][a]
BD-c1	1.2
ETV	0.94

[a] Data were obtained by standard ELISA, and values are the mean of two parallel determinations following the kit protocol.

Molecular simulation analysis

The new compounds were designed on the basis of piperidine-substituted DAPY derivatives (compound **1**) and piperidine-substituted triazine derivatives (compound **2**). To determine the binding mode of the newly synthesized compounds, molecular simulation studies were carried out. The most promising compounds **BD-c1** and **BD-e2**, as well as lead compounds **1** and **2**, were docked into the non-nucleoside inhibitor binding pocket (NNIBP) of wild-type HIV-1 RT (PDB code: 3M8Q)[5] using the software package Surflex-Dock SYBYL-X 1.1. Default parameters were used as described in the SYBYL-X 1.1 manual, unless otherwise specified; this is illustrated in detail in the Experimental Section.

It is apparent that the new compounds **BD-c1** and **BD-e2** can bind the NNIBP in a manner similar to that of lead compounds **1** and **2**, as observed by superimposition of the docked conformations (Figure 3a). The Ar groups of the four compounds were oriented directly into the protein–solvent interface, and in particular, the polar hydrophilic substituents at the *para* position extend to the exterior water (Figure 3b). Detailed analysis of the docking results revealed the following notable features, which were also observed for the lead compounds **1** and **2** as well as other DAPY compounds:[13] 1) The 4-cyano-2,6-dimethylphenyl group of **BD-c1** and the mesityl moiety of **BD-e2** fit into a sub-pocket formed by hydrophobic aromatic residues Tyr181, Tyr188, Phe227, and Trp229. Looking closely at this part, it is clear that the 2,4,6-position-substituted phenyl group is parallel to the side chain phenyl group of Tyr181 or Tyr188, exhibiting π–π interactions, and is perpendicular to the ring planes of Phe227 and the highly conserved Trp229, forming interesting CH–π interactions. 2) The nitro group and the –NH– linker of compounds **BD-c1** and **BD-e2** form two hydrogen bonds with the backbone carbonyl and α-amino group of Lys101, which are able to decrease the binding free energy for inhibitors (Figure 3c, d). Additionally, the $-SO_2Me$ group of **BD-c1** could form a hydrogen bond with Val106, and the $-SO_2NH_2$ group of **BD-e2** forms hydrogen bonds with Val106 and Lys104 (Figure 3c, d), which are located around the protein–solvent interface and could improve stability of RT–inhibitor complex. However, the new compounds display less potent activity against the resistant K103N + Y181C mutant strain of HIV-1 because of the following reason according to the analysis above. The new compounds form two hydrogen bonds with the Lys101 backbone, whereas lead compound **1** forms another hydrogen bond with the Lys103 backbone through a water bridge, and this hydrogen bond interaction with main chain is unlikely to be disrupted by side chain mutations.

Overall, the binding model analysis supports our initial design hypothesis of introducing a nitro group as a potential hydrogen bond receptor in place of the N atom of pyrimidine or triazine rings. Further structural optimizations will consider these aspects.

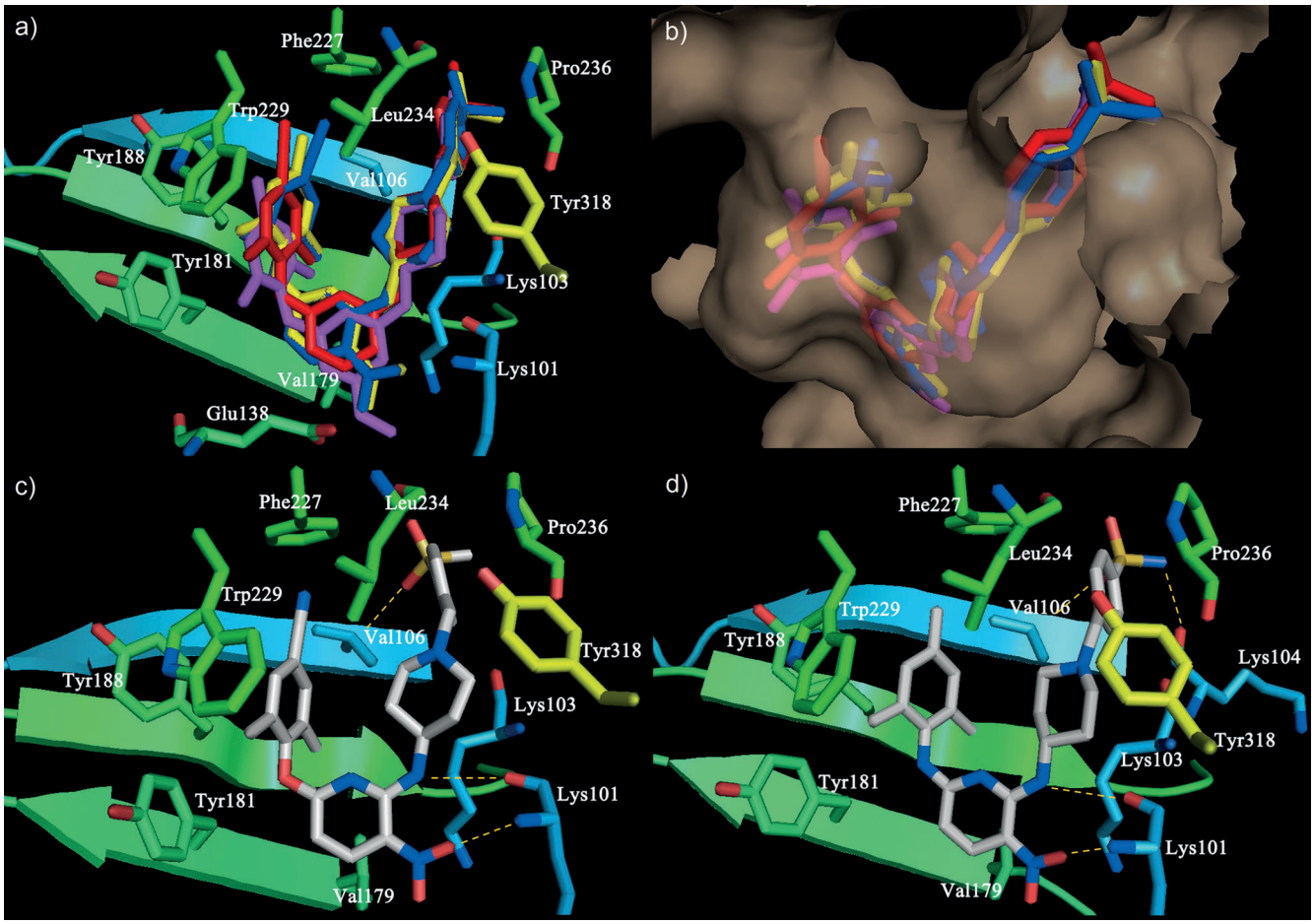


Figure 3. Predicted binding modes and docking poses (PDB code: 3M8Q) shown by PyMOL:[14] a) superimposition of the docked conformations of compounds **1** (red), **2** (magenta), **BD-c1** (blue), and **BD-e2** (yellow); b) superimposition of the docked conformations in transparent surface mode; c) compound **BD-c1**; d) compound **BD-e2**. Hydrogen bonds are indicated with dashed lines in yellow, and hydrogen atoms are omitted for clarity.

Conclusions

In summary, we designed and synthesized a series of piperidine-linked pyridine analogues to continue our research into the discovery of more active and less toxic HIV-1 NNRTIs. From a chemical point of view, the preparation of these compounds is particularly simple and was carried out via an expeditious route. The screening results in MT-4 cells indicate that most compounds show good activity against wild-type HIV-1 in the nanomolar concentration range and moderate activity against the K103N+Y181C resistant mutant strain of HIV-1, with EC_{50} values in the low-micromolar concentration range. Compound **BD-c1** ($EC_{50}=10$ nM, $CC_{50}\geq 146$ μM, $SI\geq 14126$) shows lower cytotoxicity and higher selectivity than the newly FDA-approved drug ETV, and **BD-e2** ($EC_{50}=5.1$ nM) shows better antiviral efficacy than the four reference drugs NVP, DLV, EFV, and AZT. Compounds **BD-c1** and **BD-e2** could be further developed as lead anti-HIV-1 agents. In addition, preliminary SAR studies and molecular simulations were performed to analyze the interactions between these analogues and HIV-1 RT. Further studies are ongoing in our laboratories and will be reported in due course.

Experimental Section

Chemistry

All melting points were determined on a micromelting point apparatus and are uncorrected. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a Brucker AV400 NMR spectrometer in the indicated solvents. Chemical shifts (δ) are reported with TMS as internal reference for ^{1}H NMR spectra and [D_6]DMSO ($\delta=39.6$ ppm) or $CDCl_3$ ($\delta=79.6$ ppm) for ^{13}C NMR spectra. Mass spectra were taken on an LC Autosampler Standard G1313A instrument. TLC was performed on silica gel GF_{254} for TLC, and spots were visualized by iodine vapor or by irradiation with UV light (λ 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. Reagents purchased from commercial suppliers were used without further purification.

General procedure for the synthesis of title compounds BD-a1–a4 and BD-c1–c4

(6-Chloro-3-nitropyridin-2-yl)-(1-Boc-piperidin-4-yl)amine (S-2): Na_2CO_3 (0.21 g, 2.0 mmol) and 4-amino-1-Boc-piperidine (0.20 g, 1.0 mmol) were slowly added to a solution of 2,6-dichloro-3-nitro-

pyridine (**S-1**, 0.19 g, 1.0 mmol) in EtOH (10 mL) at 0 °C. The mixture was stirred at room temperature overnight, and the solvent turned yellow. After removal of the solvent under reduced pressure, H_2O (20 mL) was added, and the mixture was extracted with EtOAc (2×10 mL). Combined organic layers were washed with a saturated solution of NaCl (10 mL) and dried over anhydrous Na_2SO_4. Purification on silica gel gave **2** as a yellow solid. Yield: 85%; TLC R_f (EtOAc/petroleum ether (PE) 1:8) = 0.32; mp: 128–130 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.36 (d, 1H, PyH, J = 8.60 Hz), 8.28 (d, 1H, J = 7.28 Hz), 6.63 (d, 1H, PyH, J = 8.64 Hz), 4.30–4.37 (m, 1H), 4.07 (d, 2H, J = 11.64 Hz), 3.02 (t, 2H, J = 11.80 Hz), 2.06 (d, 2H, J = 11.64 Hz), 1.50–1.54 (m, 2H), 1.48 ppm (s, 9H, 3×CH_3); ESIMS: m/z 357.3 $[M+H]^+$, 374.4 $[M+NH_4]^+$, 379.4 $[M+Na]^+$, 301.4 $[(M-56)+H]^+$.

(6-Mesityloxy-3-nitropyridin-2-yl)-(1-Boc-piperidin-4-yl)amine (S-3): Cs_2CO_3 (2.9 g, 9.0 mmol) was slowly added to a solution of 2,4,6-trimethylphenol (0.82 g, 6.0 mmol) in dioxane (10 mL). The mixture was stirred at room temperature for 15 min, and then intermediate **S-2** (0.71 g, 2.0 mmol) was added. The reaction mixture was held a reflux for 7 h, after which the solvent was removed under reduced pressure. H_2O (10 mL) was added to the resulting residue, and the solution was extracted with EtOAc (2×10 mL). The combined organic layers were washed with a saturated solution of NaCl (10 mL) and subsequently dried over anhydrous Na_2SO_4. Purification by flash column chromatography afforded intermediate **S-3** as a yellow solid. Yield: 66%; TLC R_f (EtOAc/PE 1:8, run two times) = 0.54; mp: 74–76 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.39 (d, 1H, PyH, J = 9.04 Hz), 8.23 (d, 1H, J = 6.32 Hz), 6.78 (s, 2H, PhH), 6.24 (d, 1H, PyH, J = 9.04 Hz), 3.91 (brs, 2H), 3.49–3.53 (m, 1H), 2.52 (m, 2H), 2.29 (s, 3H, CH_3), 2.05 (s, 6H, 2×CH_3), 1.70–1.72 (m, 3H), 1.50–1.54 (m, 2H), 1.45 ppm (s, 9H, 3×CH_3); ESIMS: m/z 457.6 $[M+H]^+$, 401.5 $[(M-56)+H]^+$.

(6-(4-Cyano-2,6-dimethylphenoxy)-3-nitropyridin-2-yl)-(1-Boc-piperidin-4-yl)amine (S-3′): 4-Hydroxy-3,5-dimethylbenzonitrile (0.22 g, 1.5 mmol), Cs_2CO_3 (0.80 g, 2.5 mmol), and intermediate **S-2** (0.17 g, 0.48 mmol) were used, following the same procedure as described for the preparation of **S-3**, to give **S-3′** as a yellow solid. Yield: 72%; TLC R_f (EtOAc/PE 1:3) = 0.30; mp: 182–184 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.49 (d, 1H, PyH, J = 9.00 Hz), 8.28 (d, 1H, J = 6.64 Hz), 7.44 (s, 2H, PhH), 6.35 (d, 1H, PyH, J = 9.00 Hz), 3.92 (d, 2H, J = 12.12 Hz), 3.38–3.45 (m, 1H), 2.55 (m, 2H), 2.15 (s, 6H, 2×CH_3), 1.67 (d, 2H, J = 10.20 Hz), 1.46 ppm (s, 9H, 3×CH_3); ESIMS: m/z 468.5 $[M+H]^+$, 485.7 $[M+NH_4]^+$, 412.5 $[(M-56)+H]^+$, 368.4 $[(M-100)+H]^+$.

6-(Mesityloxy)-3-nitro-*N*-(piperidin-4-yl)pyridin-2-amine (S-4): TFA (2.6 mL, 35 mmol) was added dropwise under stirring to a solution of intermediate **S-3** (2.0 g, 4.4 mmol) in CH_2Cl_2 (10 mL) at room temperature, and the mixture was stirred overnight. After removal of the solvent under reduced pressure, H_2O (10 mL) was added, and the mixture was neutralized with 2 N $NaOH_{(aq)}$ to pH 8. After extraction with EtOAc (2×10 mL), the combined organic layers were dried over anhydrous Na_2SO_4, filtered, and after evaporation of the solvent the product **S-4** was obtained as a yellow solid. Yield: 97%; TLC R_f (MeOH/CH_2Cl_2 1:15, add 1 drop of Et_3N) = 0.67; mp: 246–248 °C; ESIMS: m/z 357.4 $[M+H]^+$.

6-(4-Cyano-2,6-dimethylphenoxy)-3-nitro-*N*-(piperidin-4-yl)pyridin-2-amine (S-4′): Intermediate **S-3′** (0.20 g, 0.43 mmol) and TFA (0.50 mL, 6.7 mmol) were combined, following the same procedure as used in the preparation of **S-4** to give **S-4′** as a yellow solid. Yield: 94%; TLC R_f (MeOH/CH_2Cl_2 1:15, add 1 drop of Et_3N) = 0.25; mp: 222–224 °C; ESIMS: m/z 368.4 $[M+H]^+$.

Title compounds BD-a1–a4 and BD-c1–c4: Intermediate **S-4** (or **S-4′**, 0.5 mmol) was dissolved in anhydrous acetone (10 mL) in the presence of anhydrous K_2CO_3 (0.14 g, 1.0 mmol) at 0 °C, followed by the addition of appropriately substituted benzyl chloride (or 4-picolyl chloride hydrochloride, 0.6 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and H_2O (20 mL) was added. After extraction with EtOAc (2×10 mL), the organic phase was washed with a saturated solution of NaCl (10 mL) and dried over anhydrous Na_2SO_4. The product was further purified by flash column chromatography to afford title compounds **BD-a1–a4** and **BD-c1–c4**.

6-(Mesityloxy)-*N*-(1-(4-(methylsulfonyl)benzyl)piperidin-4-yl)-3-nitropyridin-2-amine (BD-a1): Yellow solid, yield: 64%; TLC R_f (EtOAc/PE 1:2) = 0.15; mp: 195–197 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.40 (d, 1H, PyH, J = 9.04 Hz), 8.27 (d, 1H, J = 6.24 Hz), 7.90 (d, 2H, PhH, J = 8.24 Hz), 7.53 (d, 2H, PhH, J = 8.24 Hz), 6.87 (s, 2H, PhH), 6.22 (d, 1H, PyH, J = 9.00 Hz), 3.52 (s, 2H, CH_2), 3.47 (brs, 1H), 3.06 (s, 3H, CH_3), 2.68 (d, 2H, J = 10.80 Hz), 2.30 (s, 3H, CH_3), 2.04 (s, 6H, 2×CH_3), 1.87 (d, 2H, J = 9.60 Hz), 1.74 (d, 2H, J = 10.80 Hz), 1.48 ppm (d, 2H, J = 9.92 Hz); ESIMS: m/z 525.5 $[M+H]^+$.

4-((4-(6-(Mesityloxy)-3-nitropyridin-2-ylamino)piperidin-1-yl)methyl)benzenesulfonamide (BD-a2): Yellow solid, yield: 58%; TLC R_f (EtOAc/PE 1:1) = 0.27; mp: 289–291 °C; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 8.44 (d, 1H, PyH, J = 9.04 Hz), 8.19 (d, 1H, J = 6.52 Hz), 7.79 (d, 2H, PhH, J = 8.12 Hz), 7.46 (d, 2H, PhH, J = 7.96 Hz), 7.32 (s, 2H), 6.93 (s, 2H, PhH), 6.42 (d, 1H, PyH, J = 9.00 Hz), 3.45 (s, 2H, CH_2), 3.26 (brs, 1H), 2.62 (d, 2H, J = 10.60 Hz), 2.27 (s, 3H, CH_3), 1.99 (s, 6H, 2×CH_3), 1.64–1.69 (m, 2H), 1.58 (d, 2H, J = 10.72 Hz), 1.41–1.46 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.47, 152.00, 148.12, 143.23, 143.16, 140.05, 134.91, 130.03, 129.45, 129.30, 126.08, 122.82, 99.64, 61.94, 52.27, 49.62, 31.22, 20.84, 16.34 ppm; ESIMS: m/z 526.3 $[M+H]^+$, 548.5 $[M+Na]^+$.

4-((4-(6-(Mesityloxy)-3-nitropyridin-2-ylamino)piperidin-1-yl)methyl)benzamide (BD-a3): Yellow solid, yield: 71%; TLC R_f (EtOAc) = 0.31; mp: 221–223 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.40 (d, 1H, PyH, J = 9.00 Hz), 8.27 (d, 1H, J = 6.36 Hz), 7.78 (d, 2H, PhH, J = 8.16 Hz), 7.41 (d, 2H, PhH, J = 8.04 Hz), 6.86 (s, 2H, PhH), 6.22 (d, 1H, PyH, J = 9.04 Hz), 6.07 (brs, 1H), 5.70 (brs, 1H), 3.50 (s, 2H, CH_2), 3.44 (brs, 1H), 2.69 (d, 2H, J = 11.56 Hz), 2.30 (s, 3H, CH_3), 2.04 (s, 6H, 2×CH_3), 1.83–1.88 (m, 2H), 1.73 (d, 2H, J = 10.96 Hz), 1.43–1.51 ppm (m, 2H); ESIMS: m/z 490.2 $[M+H]^+$, 512.2 $[M+Na]^+$.

6-(Mesityloxy)-3-nitro-*N*-(1-(pyridin-4-ylmethyl)piperidin-4-yl)-pyridin-2-amine (BD-a4): Yellow oil, yield: 61%; TLC R_f (EtOAc/PE 3:1) = 0.22; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.55 (d, 2H, PyH, J = 5.76 Hz), 8.40 (d, 1H, PyH, J = 9.00 Hz), 8.27 (d, 1H, J = 6.20 Hz), 7.28 (d, 2H, PyH, J = 5.76 Hz), 6.86 (s, 2H, PhH), 6.23 (d, 1H, PyH, J = 9.04 Hz), 3.49 (s, 2H, CH_2), 2.69 (d, 2H, J = 11.68 Hz), 2.29 (s, 3H, CH_3), 2.04 (s, 6H, 2×CH_3), 1.87–1.92 (m, 2H), 1.75 (d, 2H, J = 11.48 Hz), 1.45–1.54 ppm (m, 2H); ESIMS: m/z 448.6 $[M+H]^+$.

3,5-Dimethyl-4-(6-(1-(4-(methylsulfonyl)benzyl)piperidin-4-ylamino)-5-nitropyridin-2-yloxy)benzonitrile (BD-c1): Yellow solid, yield: 62%; TLC R_f (EtOAc/PE 1:1) = 0.22; mp: 208–210 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.48 (d, 1H, PyH, J = 8.92 Hz), 8.25 (d, 1H, J = 6.52 Hz), 7.90 (d, 2H, PhH, J = 8.32 Hz), 7.55 (d, 2H, PhH, J = 8.28 Hz), 7.42 (s, 2H, PhH), 6.35 (d, 1H, PyH, J = 8.92 Hz), 3.54 (s, 2H, CH_2), 3.26–3.29 (m, 1H), 3.07 (s, 3H, CH_3), 2.68 (d, 2H, J = 11.76 Hz), 2.14 (s, 6H, 2×CH_3), 1.78–1.83 (m, 2H), 1.66 (d, 2H, J = 10.68 Hz), 1.40–1.50 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.53, 154.07, 151.91, 145.19, 139.86, 139.27, 132.86, 132.15, 129.68, 127.42, 123.39, 118.57, 109.38, 98.86, 62.20, 52.06, 48.99,

44.49, 31.51, 16.31 ppm; ESIMS: *m/z* 536.4 $[M+H]^+$, 558.4 $[M+Na]^+$; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{27}H_{30}N_5O_5S$: 536.1962, found: 536.1954.

4-((4-(6-(4-Cyano-2,6-dimethylphenoxy)-3-nitropyridin-2-ylamino)piperidin-1-yl)methyl)benzenesulfonamide (BD-c2): Yellow solid, yield: 55%; TLC R_f (EtOAc/PE 1:1) = 0.17; mp: 225–227 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.47 (d, 1H, PyH, *J* = 8.92 Hz), 8.24 (d, 1H, *J* = 6.60 Hz), 7.79 (d, 2H, PhH, *J* = 8.36 Hz), 7.47 (d, 2H, PhH, *J* = 8.32 Hz), 7.40 (s, 2H, PhH), 6.34 (d, 1H, PyH, *J* = 9.00 Hz), 4.93 (s, 2H), 3.54 (s, 2H, CH_2), 3.19–3.26 (m, 1H), 2.68 (d, 2H, *J* = 11.84 Hz), 2.13 (s, 6H, 2×CH_3), 1.76–1.81 (m, 2H), 1.65 (d, 2H, *J* = 10.20 Hz), 1.39–1.48 ppm (m, 2H); ESIMS: *m/z* 537.4 $[M+H]^+$, 559.4 $[M+Na]^+$.

4-((4-(6-(4-Cyano-2,6-dimethylphenoxy)-3-nitropyridin-2-ylamino)piperidin-1-yl)methyl)benzamide (BD-c3): Yellow solid, yield: 74%; TLC R_f (EtOAc/PE 3:1) = 0.19; mp: 151–153 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.47 (d, 1H, PyH, *J* = 8.88 Hz), 8.24 (d, 1H, *J* = 6.48 Hz), 7.80 (d, 2H, PhH, *J* = 8.16 Hz), 7.40 (d, 2H, PhH, *J* = 8.00 Hz), 7.40 (s, 2H, PhH), 6.33 (d, 1H, PyH, *J* = 8.92 Hz), 6.20 (brs, 1H), 5.65 (brs, 1H), 3.54 (s, 2H, CH_2), 3.17–3.24 (m, 1H), 2.69 (d, 2H, *J* = 11.68 Hz), 2.12 (s, 6H, 2×CH_3), 1.74–1.79 (m, 2H), 1.65 (d, 2H, *J* = 10.24 Hz), 1.43–1.48 ppm (m, 2H); ESIMS: *m/z* 501.5 $[M+H]^+$.

3,5-Dimethyl-4-(5-nitro-6-(1-(pyridin-4-ylmethyl)piperidin-4-ylamino)pyridin-2-yloxy)benzonitrile (BD-c4): Yellow solid, yield: 52%; TLC R_f (EtOAc/PE 3:1) = 0.19; mp: 142–144 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.56 (dd, 2H, PyH, J_1 = 5.96 Hz, J_2 = 1.48 Hz), 8.48 (d, 1H, PyH, *J* = 8.92 Hz), 8.27 (d, 1H, *J* = 6.36 Hz), 7.42 (s, 2H, PhH), 7.29 (d, 2H, PyH, *J* = 5.76 Hz), 6.34 (d, 1H, PyH, *J* = 9.00 Hz), 3.48 (s, 2H, CH_2), 3.28–3.30 (m,1H), 2.70 (d, 2H, *J* = 11.68 Hz), 2.13 (s, 6H, 2×CH_3), 1.79–1.85 (m, 2H), 1.67 (d, 2H, *J* = 10.84 Hz), 1.44–1.53 ppm (m, 2H); ESIMS: *m/z* 459.6 $[M+H]^+$.

General procedure for the synthesis of title compounds BD-b1–b4 and BD-d1–d4

6-(Mesityloxy)-N^2-(1-Boc-piperidin-4-yl)pyridine-2,3-diamine (S-5): MeOH (10 mL) was added to a mixture of intermediate **S-3** (0.15 g, 0.33 mmol) and palladium on charcoal (10%, 7.5 mg), and this was kept under H_2 atmosphere. The reaction was stirred at room temperature overnight. Pd-C was removed by filtration, and the solvent was removed under reduced pressure. The gray residue (**S-5**) was dried over anhydrous Na_2SO_4. Yield: 98%; TLC R_f (EtOAc/PE 1:1) = 0.34; mp: 189–191 °C; ESIMS: *m/z* 427.6 $[M+H]^+$.

6-(4-Cyano-2,6-dimethylphenoxy)-N^2-(1-Boc-piperidin-4-yl)pyridine-2,3-diamine (S-5′): Intermediate **S-3′** (0.20 g, 0.43 mmol) and Pd-C (10%, 10 mg) were used, following the procedure as described for the preparation of intermediate **S-5** to give intermediate **S-5′** as a gray solid. Yield: 95%; TLC R_f (EtOAc/PE 1:1) = 0.19; mp: 182–184 °C; ESIMS: *m/z* 438.6 $[M+H]^+$, 382.6 $[(M-56)+H]^+$.

6-(Mesityloxy)-N^2-(piperidin-4-yl)pyridine-2,3-diamine (S-6): Intermediate **S-5** (0.20 g, 0.47 mmol) and TFA (0.50 mL, 6.7 mmol) were used, following the procedure as described for the preparation of intermediate **S-4** to give intermediate **S-6** as a gray solid. Yield: 95%; TLC R_f (MeOH/CH_2Cl_2 1:10, add 1 drop of Et_3N) = 0.67; mp: 152–154 °C; ESIMS: *m/z* 327.6 $[M+H]^+$.

6-(4-Cyano-2,6-dimethylphenoxy)-N^2-(piperidin-4-yl)pyridine-2,3-diamine (S-6′): Intermediate **S-5′** (0.60 g, 1.4 mmol) and TFA (1.0 mL, 13.5 mmol) were used, following the procedure as described for the preparation of intermediate **S-4** to give intermediate **S-6′** as a gray solid. Yield: 96%; TLC R_f (MeOH/CH_2Cl_2 1:5, add 1 drop of Et_3N) = 0.14; mp: 200–202 °C; ESIMS: *m/z* 338.6 $[M+H]^+$.

Title compounds BD-b1–b4 and BD-d1–d4: Intermediate **S-6** (or **S-6′**, 0.5 mmol) was dissolved in anhydrous acetone (10 mL) in the presence of anhydrous K_2CO_3 (0.14 g, 1.0 mmol) at 0 °C, followed by addition of the appropriately substituted benzyl chloride (or 4-picolyl chloride hydrochloride, 0.6 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and H_2O (20 mL) was added. After extraction with EtOAc (2×10 mL), the organic phase was washed with a saturated solution of NaCl (10 mL) and dried over anhydrous Na_2SO_4. Further purification was done by flash column chromatography to afford title compounds **BD-b1–b4** and **BD-d1–d4**.

6-(Mesityloxy)-N^2-(1-(4-(methylsulfonyl)benzyl)piperidin-4-yl)pyridine-2,3-diamine (BD-b1): Gray solid, yield: 43%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.35; mp: 81–83 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.90 (d, 2H, PhH, *J* = 8.28 Hz), 7.57 (d, 2H, PhH, *J* = 8.12 Hz), 6.84 (s, 2H, PhH), 6.82 (d, 1H, PyH, *J* = 8.12 Hz), 5.68 (d, 1H, PyH, *J* = 7.92 Hz), 4.27 (d, 1H, *J* = 7.00 Hz), 3.69–3.73 (m, 1H), 3.60 (s, 2H, CH_2), 3.06 (s, 3H, CH_3), 2.78 (d, 4H, *J* = 11.28 Hz), 2.27 (s, 3H, CH_3), 2.14 (d, 2H, *J* = 11.00 Hz), 2.08 (s, 6H, 2×CH_3), 1.97 (d, 2H, *J* = 10.68 Hz), 1.44–1.51 ppm (m, 2H); ESIMS: *m/z* 495.3 $[M+H]^+$.

4-((4-(3-Amino-6-(mesityloxy)pyridin-2-ylamino)piperidin-1-yl)methyl)benzenesulfonamide (BD-b2): Gray solid, yield: 46%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.14; mp: 225–227 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.90 (d, 2H, PhH, *J* = 8.28 Hz), 7.56 (d, 2H, PhH, *J* = 8.00 Hz), 6.84 (s, 2H, PhH), 6.82 (d, 1H, PyH, *J* = 7.84 Hz), 5.69 (d, 1H, PyH, *J* = 7.90 Hz), 4.87 (brs, 2H), 4.31(brs, 1H), 3.73 (brs, 1H), 3.65 (s, 2H, CH_2), 2.84 (brs, 2H), 2.27 (s, 3H, CH_3), 2.18 (m, 2H), 2.07 (s, 6H, 2×CH_3), 1.99 ppm (d, 2H, *J* = 11.64 Hz); ESIMS: *m/z* 496.2 $[M+H]^+$.

4-((4-(3-Amino-6-(mesityloxy)pyridin-2-ylamino)piperidin-1-yl)methyl)benzamide (BD-b3): Gray solid, yield: 51%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.12; mp: 174–176 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.78 (d, 2H, PhH, *J* = 8.12 Hz), 7.43 (d, 2H, PhH, *J* = 8.12 Hz), 6.84 (s, 2H, PhH), 6.81 (d, 1H, PyH, *J* = 7.84 Hz), 6.08 (brs, 1H), 5.70 (d, 1H, PyH, *J* = 7.92 Hz), 5.62 (brs, 1H), 4.27 (d, 1H, *J* = 4.44 Hz), 3.69 (d, 1H, *J* = 4.28 Hz), 3.61 (s, 2H, CH_2), 2.83 (d, 2H, *J* = 10.32 Hz), 2.27 (s, 3H, CH_3), 2.10–2.13 (m, 2H), 2.07 (s, 6H, 2×CH_3), 1.97 (d, 2H, *J* = 10.64 Hz), 1.43–1.52 ppm (m, 2H); ESIMS: *m/z* 460.2 $[M+H]^+$.

6-(Mesityloxy)-N^2-(1-(pyridin-4-ylmethyl)piperidin-4-yl)pyridine-2,3-diamine (BD-b4): Gray solid, yield: 47%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.12; mp: 131–133 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.55 (d, 2H, PyH, *J* = 4.52 Hz), 7.30 (d, 2H, PyH, *J* = 5.48 Hz), 6.84 (s, 2H, PhH), 6.82 (d, 1H, PyH, *J* = 7.88 Hz), 5.70 (d, 1H, PyH, *J* = 7.96 Hz), 4.27 (d, 1H, *J* = 6.48 Hz), 3.67–3.71 (m, 1H), 3.52 (s, 2H, CH_2), 2.78 (d, 2H, *J* = 11.24 Hz), 2.27 (s, 3H, CH_3), 2.12 (m, 2H), 2.08 (s, 6H, 2×CH_3), 1.97 (d, 2H, *J* = 11.48 Hz), 1.45–1.53 ppm (m, 2H); ESIMS: *m/z* 418.6 $[M+H]^+$, 209.9 $[(M+2)/2]^+$.

4-(5-Amino-6-(1-(4-(methylsulfonyl)benzyl)piperidin-4-ylamino)-pyridin-2-yloxy)-3,5-dimethylbenzonitrile (BD-d1): Gray solid, yield: 44%; TLC R_f (MeOH/CH_2Cl_2 1:12) = 0.16; mp: 193–195 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.90 (d, 2H, PhH, *J* = 8.28 Hz), 7.57 (d, 2H, PhH, *J* = 7.88 Hz), 7.35 (s, 2H, PhH), 6.91 (d, 1H, PyH, *J* = 7.92 Hz), 5.98 (d, 1H, PyH, *J* = 7.84 Hz), 4.20 (d, 1H, *J* = 6.36 Hz), 3.57 (s, 2H, CH_2), 3.26–3.30 (m, 1H), 3.07 (s, 3H, CH_3), 2.86 (brs, 2H), 2.73 (d, 2H, *J* = 9.76 Hz), 2.13 (s, 6H, 2×CH_3), 1.90 (d, 2H, *J* = 10.88 Hz), 1.78 (d, 2H, *J* = 11.40 Hz), 1.38 ppm (d, 2H, *J* = 10.88 Hz); ESIMS: *m/z* 506.4 $[M+H]^+$.

4-((4-(3-Amino-6-(4-cyano-2,6-dimethylphenoxy)pyridin-2-ylamino)piperidin-1-yl)methyl)benzenesulfonamide (BD-d2): Gray solid, yield: 46%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.21; mp: 224–226 °C; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 7.79 (d, 2H, PhH, J = 8.28 Hz), 7.57 (s, 2H, PhH), 7.48 (s, 2H), 7.32 (s, 2H), 6.77 (d, 1H, PyH, J = 7.80 Hz), 5.94 (d, 1H, PyH, J = 7.80 Hz), 5.42 (brs, 1H), 4.34 (brs, 2H), 3.45 (s, 2H, CH_2), 3.02 (brs, 1H), 2.86 (brs, 2H), 2.62 (brs, 2H), 2.05 (s, 6H, 2×CH_3), 1.59–1.67 ppm (m, 4H); ESIMS: *m/z* 507.1 $[M+H]^+$, 529.1 $[M+Na]^+$.

4-((4-(3-Amino-6-(4-cyano-2,6-dimethylphenoxy)pyridin-2-ylamino)piperidin-1-yl)methyl)benzamide (BD-d3): Gray solid, yield: 53%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.17; mp: 165–167 °C; ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 7.92 (s, 1H), 7.83 (d, 2H, PhH, J = 7.84 Hz), 7.57 (s, 2H, PhH), 7.35 (d, 2H, PhH, J = 7.20 Hz), 7.30 (s, 1H), 6.77 (d, 1H, PyH, J = 7.84 Hz), 5.94 (d, 1H, PyH, J = 7.80 Hz), 5.40 (d, 1H, J = 5.32 Hz), 4.33 (brs, 2H), 3.42 (s, 2H, CH_2), 3.02 (brs, 1H), 2.65 (d, 1H, J = 8.32 Hz), 2.05 (s, 6H, 2×CH_3), 1.58–1.66 ppm (m, 4H); ESIMS: *m/z* 471.6 $[M+H]^+$.

4-(5-Amino-6-(1-(pyridin-4-ylmethyl)piperidin-4-ylamino)pyridin-2-yloxy)-3,5-dimethylbenzonitrile (BD-d4): Gray solid, yield: 48%; TLC R_f (MeOH/CH_2Cl_2 1:10) = 0.24; mp: 158–160 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.55 (dd, 2H, PyH, J_1 = 5.92 Hz, J_2 = 1.44 Hz), 7.36 (s, 2H, PhH), 7.28 (d, 2H, PyH, J = 5.92 Hz), 6.91 (d, 1H, PyH, J = 7.84 Hz), 5.94 (d, 1H, PyH, J = 7.88 Hz), 4.20 (d, 2H, J = 6.84 Hz), 3.47 (s, 2H, CH_2), 3.25–3.33 (m, 1H), 2.71 (d, 2H, J = 11.84 Hz), 2.13 (s, 6H, 2×CH_3), 1.86–1.1.92 (m, 2H), 1.77 ppm (d, 2H, J = 11.96 Hz); ESIMS: *m/z* 429.5 $[M+H]^+$.

General procedure for the synthesis of title compounds BD-e1–e4

N^6-Mesityl-N^2-(1-Boc-piperidin-4-yl)-3-nitropyridine-2,6-diamine (S-7): A mixture of 2,4,6-trimethylaniline (0.27 g, 2.0 mmol) and intermediate **S-2** (0.36 g, 1.0 mmol) was heated at 130 °C overnight. After addition of H_2O, the reaction mixture was extracted with EtOAc (2×10 mL). The combined organic layers were washed with a saturated solution of NaCl (10 mL) and dried over anhydrous Na_2SO_4. Purification on silica gel gave **S-7** as a yellow solid. Yield: 65%; TLC R_f (EtOAc/PE 1:5) = 0.41; mp: 177–179 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.72 (s, 1H, PyH), 8.14 (d, 1H, J = 8.00 Hz), 6.96 (s, 2H, PhH), 6.51 (s, 1H), 5.37 (s, 1H), 4.34 (s, 1H), 4.05 (s, 2H), 3.03 (s, 2H), 2.32 (s, 3H, CH_3), 2.18 (s, 6H, 2×CH_3), 2.06 (s, 2H), 1.50–1.54 (m, 2H), 1.48 ppm (s, 9H, 3×CH_3); ESIMS: *m/z* 456.5 $[M+H]^+$,478.5 $[M+Na]^+$, 400.4 $[(M-56)+H]^+$.

N^6-Mesityl-3-nitro-N^2-(piperidin-4-yl)pyridine-2,6-diamine (S-8): TFA (2.6 mL, 35 mmol) was added dropwise, under stirring, to a solution of intermediate **S-7** (2.0 g, 4.4 mmol) in CH_2Cl_2 (10 mL) at room temperature and stirred overnight. After removal of the solvent under reduced pressure, H_2O (10 mL) and EtOAc (10 mL) were added. An aqueous 2 N HCl solution was added dropwise (to pH 2), and the aqueous phase was separated and subsequently neutralized with 2 N $NaOH_{(aq)}$ to pH 8 to obtain a yellow precipitate. Filtration and drying gave the product (**S-8**) as a yellow solid. Yield: 95%; TLC R_f (MeOH/CH_2Cl_2 1:10, add 1 drop of Et_3N) = 0.31; mp: 187–189 °C; ESIMS: *m/z* 356.4 $[M+H]^+$.

Title compounds BD-e1–e4: Intermediate **S-8** (0.5 mmol) was dissolved in anhydrous acetone (10 mL) in the presence of anhydrous K_2CO_3 (0.14 g, 1.0 mmol) at 0 °C, followed by addition of the appropriately substituted benzyl chloride (or 4-picolyl chloride hydrochloride, 0.6 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and H_2O (20 mL) was added, followed by extraction with EtOAc (2×10 mL). The organic phase was washed with saturated NaCl (10 mL), dried over anhydrous Na_2SO_4, and was purified by flash column chromatography to afford title compounds **BD-e1–e4**.

N^6-Mesityl-N^2-(1-(4-(methylsulfonyl)benzyl)piperidin-4-yl)-3-nitropyridine-2,6-diamine (BD-e1): Yellow solid, yield: 63%; TLC R_f (EtOAc/PE 1:1) = 0.23; mp: 106–108 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.77 (brs, 1H), 8.13 (d, 1H, PyH, J = 8.96 Hz), 7.91 (d, 2H, PhH, J = 8.12 Hz), 7.59 (d, 2H, PhH, J = 4.28 Hz), 6.95 (s, 2H, PhH), 6.51 (brs, 1H), 5.36 (brs, 1H), 4.24 (brs, 1H), 3.66 (s, 2H, CH_2), 3.06 (s, 3H, CH_3), 2.83 (brs, 2H), 2.31 (s, 3H, CH_3), 2.17 (s, 6H, 2×CH_3), 2.02–2.12 (m, 2H), 1.48–1.94 ppm (m, 4H); ESIMS: *m/z* 524.5 $[M+H]^+$, 546.3 $[M+Na]^+$.

4-((4-(6-(Mesitylamino)-3-nitropyridin-2-ylamino)piperidin-1-yl)methyl)benzenesulfonamide (BD-e2): Yellow solid, yield: 58%; TLC R_f (EtOAc/PE 1:1) = 0.11; mp: 138–140 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.75 (brs, 1H), 8.11 (d, 1H, PyH, J = 9.04 Hz), 7.88 (d, 2H, PhH, J = 8.24 Hz), 7.49 (d, 2H, PhH, J = 7.80 Hz), 6.95 (s, 2H, PhH), 6.59 (brs, 1H), 5.36 (brs, 1H), 5.18 (brs, 2H), 4.24 (brs, 1H), 3.60 (s, 2H, CH_2), 2.79 (brs, 2H), 2.31 (s, 3H, CH_3), 2.16 (s, 6H, 2×CH_3), 1.85–2.11 (m, 4H), 1.58–1.72 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 160.89, 153.50, 143.98, 140.75, 143.16, 137.57, 136.49, 131.66, 129.54, 129.39, 127.10, 126.63, 120.44, 96.65, 62.39, 51.99, 31.83, 20.98, 18.25 ppm; ESIMS: *m/z* 525.5 $[M+H]^+$, 547.4 $[M+Na]^+$; HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{26}H_{33}N_6O_4S$: 525.2279, found: 525.2269.

4-((4-(6-(Mesitylamino)-3-nitropyridin-2-ylamino)piperidin-1-yl)methyl)benzamide (BD-e3): Yellow solid, yield: 67%; TLC R_f (EtOAc/PE 3:1) = 0.18; mp: 255–257 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.75 (brs, 1H), 8.13 (d, 1H, PyH, J = 8.88 Hz), 7.78 (d, 2H, PhH, J = 8.20 Hz), 7.44 (d, 2H, PhH, J = 7.64 Hz), 6.95 (s, 2H, PhH), 6.51 (brs, 1H), 6.07 (brs, 1H), 5.66 (brs, 1H), 5.35 (brs, 1H), 4.23 (brs, 1H), 3.60 (s, 2H, CH_2), 2.82 (brs, 2H), 2.31 (s, 3H, CH_3), 2.17 (s, 6H, 2×CH_3), 2.08 (brs, 2H), 1.71 ppm (brs, 2H); ESIMS: *m/z* 489.5 $[M+H]^+$, 511.6 $[M+Na]^+$.

N^6-Mesityl-3-nitro-N^2-(1-(pyridin-4-ylmethyl)piperidin-4-yl)pyridine-2,6-diamine (BD-e4): Yellow solid, yield: 55%; TLC R_f (EtOAc) = 0.18; mp: 228–230 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.77 (brs, 1H), 8.56 (d, 2H, PyH, J = 5.84 Hz), 8.13 (d, 1H, PyH, J = 8.60 Hz), 7.30 (d, 2H, PyH, J = 4.04 Hz), 6.95 (s, 2H, PhH), 6.54 (brs, 1H), 5.36 (brs, 1H), 4.24 (brs, 1H), 3.56 (s, 2H, CH_2), 2.82 (brs, 2H), 2.31 (s, 3H, CH_3), 2.17 (s, 6H, 2×CH_3), 2.02–2.10 (m, 2H), 1.65–1.89 ppm (m, 4H); ESIMS: *m/z* 447.5 $[M+H]^+$.

In vitro anti-HIV activity assays

The anti-HIV activity and cytotoxicity of the compounds were evaluated against wild-type HIV-1 strain III_B, a double RT mutant (K103N + Y181C) HIV-1 strain, and HIV-2 strain ROD in MT-4 cell cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) method as previously described.[8,13] Briefly, 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 test compounds (final 200 μL volume per well) were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman Instruments). Untreated control HIV- and mock-infected cell samples were included for each sample. HIV-1 (III_B)[15] or HIV-2 (ROD)[16] stock (50 μL) at 100–300 $CCID_{50}$ (cell cul-

ture 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 the cytotoxicity of the test compounds. Exponentially growing MT-4 cells were centrifuged for 5 min at 220 *g*, and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells mL^{-1}, and 50 μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by MTT assay.

The MTT assay is based on the reduction of yellow-colored MTT (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase activity 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 (Infinite M1000, Tecan), at two wavelengths (540 and 690 nm). All data were calculated using the median optical density (OD) values of three wells. The 50% cytotoxic concentration (CC_{50}) is defined as the concentration of the test compound required to decrease the absorbance (OD_{540}) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

Recombinant HIV-1 RT inhibitory assays

The HIV-RT inhibition assay was performed by using an RT assay kit (Roche), and the procedure for assaying RT inhibition was performed as described in the kit protocol. Briefly, the reaction mixture consisted of template/primer complex, 2′-deoxynucleotide 5′-triphosphates (dNTPs) and reverse transcriptase (RT) in lysis buffer with or without inhibitors. After 1 h incubation at 37 °C the reaction mixture was transferred to a streptavidin-coated microtiter plate (MTP). The biotin-labeled dNTPs that are incorporated in the template through RT activity are bound to streptavidin. The unbound dNTPs were washed with wash buffer, and antidigoxigenin–peroxidase (DIG–POD) was added into the MTP. The DIG-labeled dNTPs incorporated in the template are bound to anti-DIG–POD antibody. The unbound anti-DIG–POD was washed, and the peroxide substrate (ABST) was added to the MTP. A colored reaction product was produced during cleavage of the substrate, as catalyzed by POD. The absorbance of the sample was determined at OD_{405} using a microtiter plate ELISA reader. The resulting color intensity is directly proportional to RT activity. The percent inhibitory activity of RT inhibitors was calculated by comparison with a sample lacking inhibitor. Percent inhibition values were calculated by the following: $\%\ \text{Inhibition} = 100-[(OD_{405}{}^{+\text{inhibitor}}/OD_{405}{}^{-\text{inhibitor}})\times100]$.

Molecular simulations

Molecular modeling was carried out with the Tripos molecular modeling package SYBYL-X 1.1. All the molecules for docking were built using standard bond lengths and angles from SYBYL-X 1.1/Base Builder and were then optimized using the Tripos force field. The flexible docking method, called Surflex-Dock, docks the ligand automatically into the ligand binding site of the receptor by using a protocol-based approach and an empirically derived scoring function. The protocol is a computational representation of a putative ligand that binds to the intended binding site and is a unique and essential element of the docking algorithm. The scoring function in Surflex-Dock, which contains hydrophobic, polar, repulsive, entropic, and salvation terms, was trained to estimate the dissociation constant (K_d) expressed in $-\log(K_d)^2$. Prior to docking, the protein was prepared by removing water molecules, the ligand, and other unnecessary small molecules from the crystal structure complex (PDB code: 3M8Q); simultaneously, polar hydrogen atoms were added to the protein. Surflex-Dock default settings were used for other parameters, such as the number of starting conformations per molecule, the size to expand search grid, the maximum number of rotatable bonds per molecule, and the maximum number of poses per ligand. During the docking procedure, all of the single bonds in residue side chains inside the defined RT binding pocket were regarded as rotatable or flexible, and the ligand was allowed to rotate on all single bonds and to move flexibly within the tentative binding pocket. The atomic charges were recalculated using the Kollman all-atom approach for the protein and the Gasteiger–Hückel approach for the ligand. The binding interaction energy was calculated to include van der Waals, electrostatic, and torsional energy terms defined in the Tripos force field. The 20 best-scoring ligand–protein complexes were kept for further analyses. The $-\log(K_d)^2$ values of the 20 best-scoring complexes, which represent the binding affinities of ligand with RT, ranged a wide scope of functional classes. Therefore, only the highest-scoring 3D structural model of the ligand-bound RT was chosen to define the binding interaction.

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Keywords: antiviral agents · biological activity · HIV-1 · molecular simulations · reverse transcriptase

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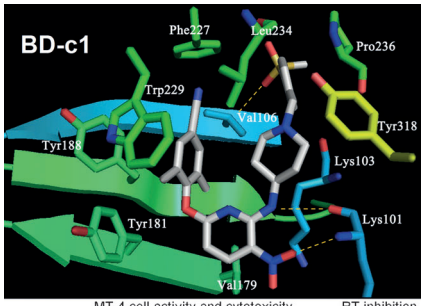
Selective and effective! Compound **BD-c1** shows impressively low cytotoxicity ($CC_{50} > 146$ μM), high selectivity (SI > 14,126), and good inhibitory activity toward HIV-1 reverse transcriptase. With qualities similar to those of the new FDA-approved drug etravirine ($CC_{50} = 28$ μM, SI = 12884), **BD-c1** is a promising lead compound for the development of even more efficacious anti-HIV drugs.



Compd	MT-4 cell activity and cytotoxicity			RT inhibition
	EC_{50} [nM]	CC_{50} [μM]	SI	IC_{50} [μM]
BD-c1	10 ± 2.5	≥146	≥14126	1.2
Etravirine	2.2 ± 0.4	28 ± 12	12884	0.94

*X. Chen, Y. Li, S. Ding, J. Balzarini, C. Pannecouque, E. De Clercq, H. Liu, X. Liu**

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Discovery of Piperidine-Linked Pyridine Analogues as Potent Non-nucleoside HIV-1 Reverse Transcriptase Inhibitors