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Design, synthesis and biological evaluation of cycloalkyl arylpyrimidines (CAPYs) as HIV-1 NNRTIs

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ABSTRACT

A series of 18 cycloalkyl arylpyrimidines (CAPYs) were designed from lead compounds diarylpyrimidines (DAPYs), synthesized and evaluated for in vitro anti-HIV activity. Among them, the compound **1p** displayed potent anti-HIV-1 activity against WT HIV-1 with an EC_{50} value of 0.055 μM and a selectivity index (SI) >7290. The preliminary structure–activity relationship (SAR) of this new series of compounds was also investigated, which enriched the SAR of diarylpyrimidines (DAPYs).

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is the causative virus of acquired immunodeficiency syndrome (AIDS). So far there is still no an effective vaccine against HIV/AIDS, and the generally adopted highly active antiretroviral therapy (HAART) has evidently reduced the mortality of HIV-infected people.[1] Reverse transcriptase (RT), an essential enzyme in the infectious life cycle of HIV, is the most important target for antiretroviral chemotherapy.[2] Nonnucleoside reverse transcriptase inhibitors (NNRTIs), known as one of the indispensable components of HAART for specifically inhibiting HIV-1 reverse transcriptase (RT), have received wide attention due to their unique antiviral potency, high specificity and low cytotoxicity.[3] However, the rapid emergence of drug resistance and serious side effects of long term clinical drugs impelled medicinal chemists to develop diverse structures of NNRTIs, such as benzophenones,[4] diaryl ethers,[5] 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymines (HEPTs),[6] dihydro-alkoxybenzyl-oxopyrimidines (DABOs),[7] diaryltriazines (DATAs),[8] and diarylpyrimidines (DAPYs).[9] Among these series, DAPYs have been regarded as one of the most successful members of the NNRTIs, in which etravirine (TMC125, Fig. 1)[10] and rilpivirine (TMC278, Fig. 1)[11] became the only two approved anti-HIV chemical entities by the U.S. Food and Drug Administration (FDA) in the past few years.

Since the wing I of the DAPY structure was confirmed as the indispensable pharmacophore, the further modifications were mainly focused on the structural diversity of the linker between the wing II and the central pyrimidine ring,[12] such as *CH_2*-DAPYs,[9a] *O*-DAPYs,[9i,13] *S*-DAPYs,[9a] *NH*-DAPYs,[9a,11] *C*(=*NOH*)-DAPYs,[9e] *CH*(*CN*)-DAPYs,[9g] *CH*(*OH*)-DAPYs[14] and *CH*(*Me*)-DAPYs.[9j] However, the modifications of the wing II are limited to different substituted phenyl or naphthyl groups,[9f,b] but not involved a cycloalkyl group. In addition, in the modification of HEPT compound MKC-442 (Fig. 1),[15] the benzyl at the *C*-6 position of pyrimidine-2,4(1*H*,3*H*)-dione was replaced by the thiocyclohexyl to yield the more potent compound TNK-6123 (Fig. 1). Recently, the *S*-DACOs (Fig. 1) reported by He et al.[16] with a *C*-6 cyclohexyl taking the place of *C*-6 phenyl of *S*-DABOs (Fig. 1), are also especially attractive for their unexpectedly potent activity against wild-type HIV-1 (WT HIV-1), among which the EC_{50} value of the most potent compound (R_1 = Et, R_2 = $PhCH_2$) is as low as 0.012 nM.

The improvement of TNK-6123 and *S*-DACOs, compared with their lead compounds MKC-442 and *S*-DABOs, may be due to the improved molecular flexibility,[15–17] which could facilitate the binding of the inhibitors to their binding sites. In view of HEPTs, DABOs, DACOs and DAPYs acting at the same RT target, we decided to examine whether the introduction of a cycloalkyl to DAPYs to

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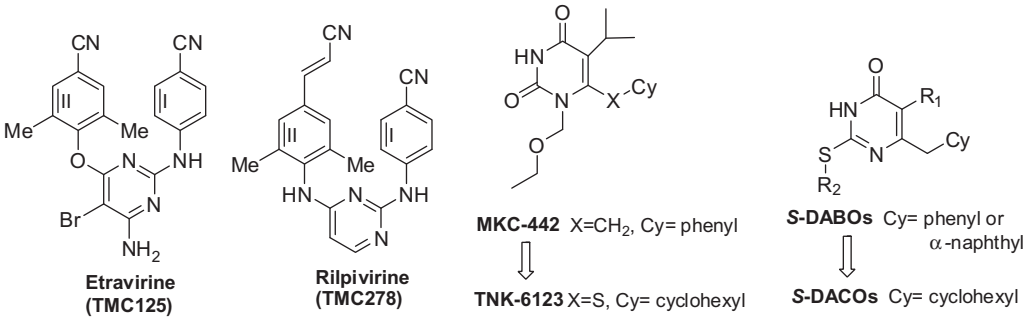


Figure 1. Structures of some NNRTIs.

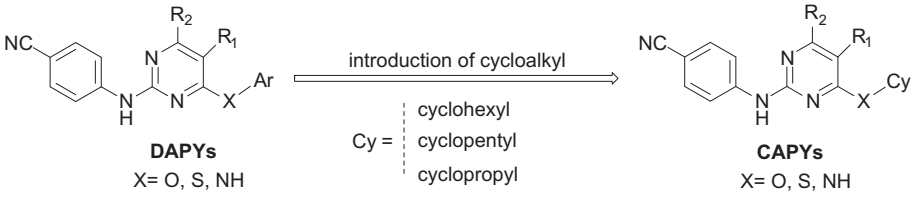


Figure 2. Analog-based design of CAPYs.

replace the phenyl could offer a novel structural scaffold (cycloalkyl arylpyrimidines, CAPYs, Fig. 2) with improved anti-HIV-1 activities. Herein we report the synthesis, anti-HIV activity, and preliminary structure–activity relationships (SARs) of these CAPYs.

2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds **1a**–**r** was outlined in Scheme 1. The 2-(methylthio)-1*H*-pyrimidin-4-ones (**7a–c**) were afforded by the methylation of 2-thioxo-2,3-dihydro-1*H*-pyrimidin-4-ones (**6a–c**), among which **6a** was a commercially available product, and **6b** as well as **6c** were readily prepared. Treatment of **7a–c** with 4-cyanoaniline in the melt condition gave 4-((4-oxo-1,4-dihydropyrimidin-2-yl)amino)benzonitriles (**8a**–**c**), which was subjected to chlorination in the presence of phosphorus oxychloride to afford 4-((4-chloropyrimidin-2-yl)amino)benzonitriles (**9a**–**c**). Target molecules **1a–r** were prepared via nucleophilic reaction of **9a–c** with different cycloalkyl alcohols, amines or thioalcohols under alkaline conditions.

2.2. Biological activity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) method[18] was used to evaluate 18 new CAPYs (**1a**–**r**) along with five FDA-approved drugs: nevirapine, zidovudine, efavirenz, delavirdine and etravirine as reference compounds. These compounds were assayed for their cytotoxicity and anti-HIV activities in MT-4 cells infected with wild-type HIV-1 (strain, III_B), double RT mutant (K103N + Y181C) HIV-1, with Lys 103 replaced by Asn and Tyr181 by Cys), as well as HIV-2 strain ROD. The results, expressed as CC_{50} (50% cytotoxic concentration), EC_{50} (50% HIV-1 cytoprotective concentration against HIV-induced cytopathogenicity) and SI (selectivity index represented by the CC_{50}/EC_{50} ratio) values, are listed in Table 1.

As shown in Table 1, some CAPYs showed moderate to potent activities against wild-type (WT) HIV-1 with EC_{50} values in the range of 6.71–0.055 μM. Among them, the compound **1p** displays good anti-HIV-1 activity against WT HIV-1 with an EC_{50} value of 0.055 μM and the greatest selectivity (SI >7290). Unfortunately, the tested compounds proved inactive against the double RT mutant virus (K103N + Y181C) and HIV-2 strain ROD, with the exception of **1b** displaying some anti-HIV-2 activity (EC_{50} = 31.29 μM).

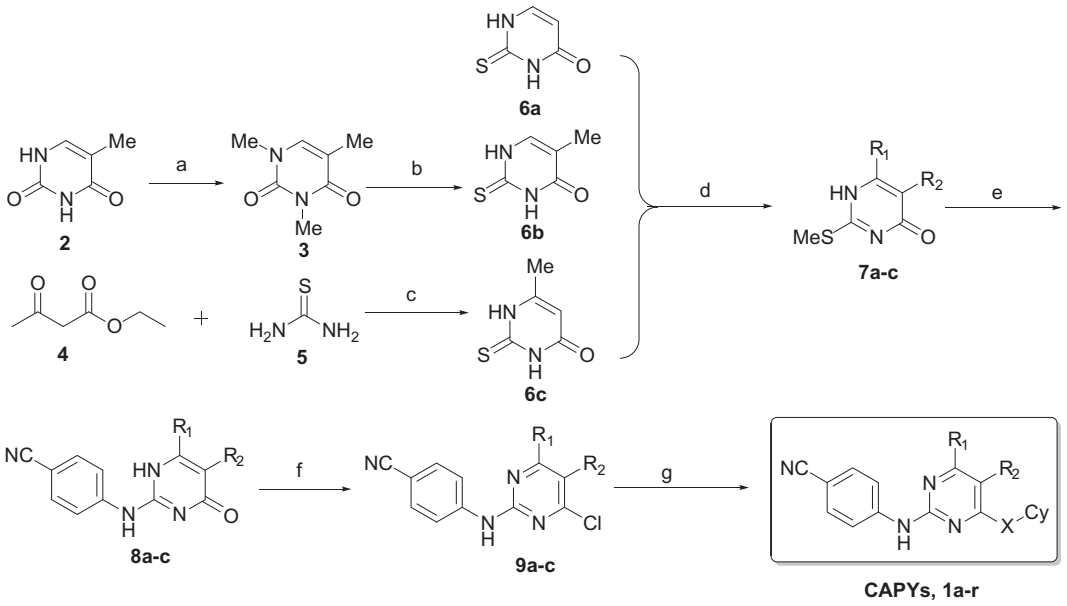
In consideration of the preferability of the linker X (O, NH or S) of CAPYs on activities against WT HIV-1, the activity sequence should be S > NH > O. The compounds **1p–r** with an S linker (*S*-CAPYs) are the most potent three compounds among 18 CAPYs. The compounds **1a–o** with a O or NH linker (*O*-CAPYs or *NH*-CAPYs) are all unsatisfactory with moderate to poor activity and low selectivity, most of which possess an SI lower than 10. The substituents R_1 and R_2 on the central pyrimidine of CAPYs seems to be harmful and needless for all *O*-CAPYs, *NH*-CAPYs and *S*-CAPYs.

As far as the cycloalkyls are concerned, for the anti-HIV-1 activity of *O*-CAPYs, the preferred cycloalkyls seem to be in the sequence of cyclohexyl > cyclopentyl; and for *NH*-CAPYs, the sequence is cyclohexyl > cyclopentyl > cyclopropyl. That is in accordance with the bulky sequence. The most potent compound **1p** is superior to the reference drug nevirapine against WT HIV-1, but 25-fold weaker than DAPY analog etravirine. Moreover, like all other CAPYs, **1p** did not inhibit the double RT mutant virus (K103N + Y181C).

In general, most of previously discovered NNRTIs are only active against HIV-1 virus and lack anti-HIV-2 activity, but this does not mean they cannot serve as HIV-2 inhibitors.[19] HIV-1 and HIV-2 reverse transcriptase are similar in structure and functionality, so it is not a surprise for **1b** to exhibit both anti-HIV-1 and some anti-HIV-2 activities. However, **1b** is not attractive because of its rather poor anti-HIV activity.

2.3. Molecular modeling

With the aim to investigate the binding mode of our newly synthesized compounds, three CAPYs, **1a**, **1p** and **1r**, were docked into the HIV-1 RT nonnucleoside binding site (NNBS) using the soft SURFLEX-DOCK SYBYL-X 1.2 (Fig. 3). Coordinates of the NNBS were taken from the crystal structure of the RT/TMC120 complex (PDB code: 1S6Q) due to structural similarity between TMC120 and CAPYs. From Fig. 3a, it could be found that the binding conformation of **1a**, **1p** and **1r** varies greatly in spite of their similar structure, especially, the orientation of 4-cyanoaniline of **1r** is even opposite to that of **1a** and **1p**. From Fig. 3b, we also found that



7, 8, 9	R_1	R_2
a	H	H
b	H	Me
c	Me	H

1	X	R_1	R_2	Cy
a	O	H	H	cyclohexyl
b	O	H	H	cyclopentyl
c	O	H	Me	cyclohexyl
d	O	H	Me	cyclopentyl
e	O	Me	H	cyclohexyl
f	O	Me	H	cyclopentyl
g	NH	H	H	cyclohexyl
h	NH	H	H	cyclopentyl
i	NH	H	H	cyclopropyl

1	X	R_1	R_2	Cy
j	NH	H	Me	cyclohexyl
k	NH	H	Me	cyclopentyl
l	NH	H	Me	cyclopropyl
m	NH	Me	H	cyclohexyl
n	NH	Me	H	cyclopentyl
o	NH	Me	H	cyclopropyl
p	S	H	H	cyclohexyl
q	S	H	Me	cyclohexyl
r	S	Me	H	cyclohexyl

Scheme 1. Synthetic route to CAPYs (**1a**–**r**). Reagents and conditions: (a) Me_2SO_4, NaOH, H_2O, rt to 50 °C; (b) NaOEt, EtOH; (c) NaOH, H_2O; (d) MeI, NaOH, H_2O, rt, 24 h; (e) 4-cyanoaniline, 180–190 °C, 10 h; (f) $POCl_3$, reflux, 0.5 h; (g) cycloalkyl alcohols or thioalcohols, NaH as alkali; or cycloalkyl amines, K_2CO_3 as alkali.

Table 1
Anti-HIV activities and cytotoxicity of compounds **1a**–**r** in MT-4 cells[a]

Compd	EC_{50}[b] [μM]			CC_{50}[c] [μM]	SI[d]
	WT (III_B)	K103N + Y181C	HIV-2		
1a	1.16 ± 0.31	>322.03	>322.03	322.03 ± 48.24	276
1b	6.71 ± 1.14	>339.61	31.29 ± 33.46	⩾339.61	⩾51
1c	6.49 ± 2.01	>31.23	>31.23	31.23 ± 2.04	5
1d	>9.99	ND[e]	>9.99	9.99 ± 1.63	<1
1e	>0.32	ND	>0.32	0.32 ± 0.03	<1
1f	>1.05	ND	>1.05	1.05 ± 0.37	<1
1g	0.96 ± 0.31	>7.33	>7.33	7.33 ± 0.92	8
1h	1.58 ± 0.36	>9.13	>9.13	9.13 ± 1.86	6
1i	>1.19	ND	>1.19	1.19 ± 0.40	<1
1j	>1.30	ND	>1.30	1.30 ± 0.17	<1
1k	>0.17	ND	>0.17	0.17 ± 0.11	<1
1l	>0.36	ND	>0.36	0.36 ± 0.02	<1
1m	>0.34	ND	>0.34	0.34 ± 0.07	<1
1n	>0.31	ND	>0.31	0.31 ± 0.01	<1
1o	>0.088	ND	>0.088	0.088 ± 0.036	<1
1p	0.055 ± 0.032	>402.68	>402.68	>402.68	>7290
1q	0.43 ± 0.40	>33.38	>33.38	33.38 ± 2.80	79
1r	0.096 ± 0.049	>192.02	>192.02	⩾192.02	⩾2006
Nevirapine	0.21 ± 0.09	3.49 ± 2.52	>15.02	>15.02	>72
Zidovudine	0.0075 ± 0.0019	0.013 ± 0.010	0.0075 ± 0.0034	⩾46.44	⩾6359
Efavirenz	0.0054 ± 0.0006	0.51	ND	>6.34	>1146
Delavirdine	0.016 ± 0.011	>4.39	ND	>4.39	>274
Etravirine	0.0022 ± 0.0004	0.034 ± 0.0046	>27.78	27.78 ± 11.53	12884

[a] Data represent the mean of at least three separate experiments.
[b] Compound concentration required to protect MT-4 cells against viral cytopathogenicity by 50%.
[c] Compound concentration that decreases the uninfected MT-4 cell viability by 50%.
[d] Selectivity index: CC_{50}/EC_{50} (WT) ratio.
[e] ND: not determined.

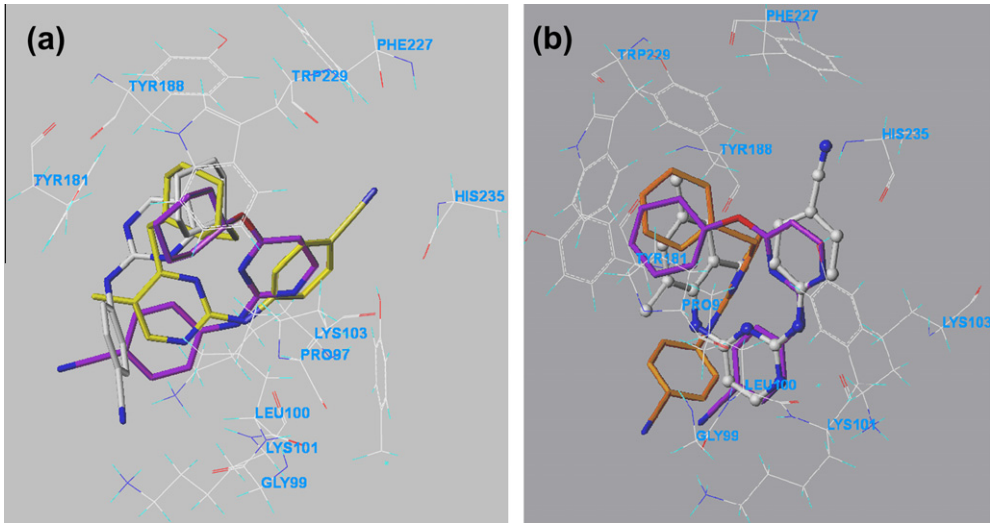


Figure 3. Model of CAPYs docking into the NNBS of HIV-RT (PDB code:1S6Q): (a) **1a** is shown in purple, **1p** in gray, **1r** in yellow; (b) **1a** is shown in purple, **1p** in orange, TMC120 in orange ball-and-stick model.

1a and **1p** do not bind in a 'horseshoe' conformation like TMC120. Although the cyclohexyls of CAPYs could also be docked in the Y181-Y188-W229 hydrophobic binding pocket, they could not form π–π interactions with these aromatic amino acid residues. Compared with TMC120, the different binding model of CAPYs may explain that that almost all the compounds exhibit no activities against the double RT mutant virus (K103N + Y181C) HIV-1.

3. Conclusions

In summary, we designed and synthesized a series of CAPYs, in which several cycloalkyl functions were introduced to replace the phenyl ring in DAPYs. Biological evaluation indicated that some CAPYs showed moderate to potent activities against wild-type (WT) HIV-1 with EC_{50} values in the range of 6.71–0.055 μM. Compound **1p** displayed good anti-HIV-1 activity against WT HIV-1 with an EC_{50} value of 0.055 μM and a selectivity index (SI) >7290. None of the compounds exhibited activity against the double RT mutant virus (K103N + Y181C). Although the molecular flexibility of CAPYs is better than that of DAPYs, the DAPYs are obviously more potent than CAPYs in anti-HIV-1 and anti-HIV-2 activities, which indicates that the rigidity of wing II is important for DAPYs. Therefore, the present research has enriched the SAR of DAPYs.

4. Experimental

4.1. Chemistry

Melting points were measured on a SGW X-1 microscopic melting-point apparatus. ^{1}H NMR and ^{13}C NMR spectra on a Bruker AV 400 MHz spectrometer were recorded in DMSO-d_6. Chemical shifts are reported in δ (ppm) units relative to the internal standard tetramethylsilane (TMS). Mass spectra were obtained on a Waters Quattro Micromass instrument using electrospray ionization (ESI) techniques or on an Agilent MS/5975 spectrometer using electron ionization (EI) techniques. Elemental analyses were performed on a Carlo Erba 1106 instrument. All chemicals and solvents used were of reagent grade and were purified and dried by standard methods before use. All air-sensitive reactions were run under a nitrogen atmosphere. All the reactions were monitored by thin layer chromatography (TLC) on pre-coated silica gel G plates at 254 nm under a UV lamp using ethyl acetate/hexane as eluent. Column chromatography separations were obtained on silica gel (300–400 mesh). The preparation of the intermediate **6b, 6c**, **7a–c**, **8a–c**, **9a–c** as well as the spectrums of **10a–r** was shown in the Supplementary data.

4.1.1. General procedure for preparation of target compounds 1a–f and 1p–r

A mixture of **9a–c** (1 mmol), cycloalkyl alcohols or cyclohexyl thiol (2 mmol), 60% sodium hydride (2.5 mmol) and anhydrous DMF (15 mL) was heated to 100 °C under stirring for 4 h. The mixture was poured into water (100 mL), and the resulting precipitates were filtered and purified by column chromatography to give products **1a–f** and **1p–r**.

4.1.2. General procedure for preparation of target compounds 1g–o

A mixture of **9a–c** (1 mmol), cycloalkyl amines (3 mmol), potassium carbonate (2 mmol) and anhydrous DMF (15 mL) was heated to 110 °C under stirring for 8 h. The mixture was poured into water (100 mL), and the resulting precipitates were filtered and purified by column chromatography to give products **1g–o**.

4.1.2.1. 4-((4-(Cyclohexyloxy)pyrimidin-2-yl)amino)benzonitrile (1a). Yield 62%; white solid; mp 190.1–191.2 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.24–2.00 (m, 10H, cyclohexyl H_{2-11}), 5.02–5.05 (m, 1H, cyclohexyl H_1), 6.33(d, J = 6.0 Hz, 1H, pyrimidine H_5), 7.70 (d, J = 8.4 Hz, 2H, Ar$H_{2,6}$), 7.94(d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 8.24 (d, J = 6.0 Hz, 1H, pyrimidine H_6), 10.02(s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.51, 24.98, 31.20, 73.86, 100.49, 102.33, 118.32, 119.54, 132.91, 144.96, 158.51, 159.08, 168.61; MS(EI) m/z 294 (M^+); Anal. calcd for $C_{17}H_{18}N_4O$: C, 69.37; H, 6.16; N, 19.03. Found: C, 69.19; H, 6.24; N 18.98.

4.1.2.2. 4-((4-(Cyclopentyloxy)pyrimidin-2-yl)amino)benzonitrile (1b). Yield 71%; white solid; mp 172.2–172.9 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.60–2.01 (m, 8H, cyclopentyl H_{2-9}), 5.41 (s, 1H, cyclopentyl H_1), 6.33 (d, J = 5.6 Hz, 1H, pyrimidine H_5), 7.71 (d, J = 8.4 Hz, 2H, Ar$H_{2,6}$), 7.96 (d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 8.24 (d, J = 5.6 Hz, 1H, pyrimidine H_6), 10.03 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.44, 32.28, 78.37, 100.50, 102.30, 118.36, 119.58, 132.95, 144.95, 158.38, 159.08,

168.88; MS(EI) *m*/*z* 280 (M^+); Anal. calcd for $C_{16}H_{16}N_4O$: C, 68.55; H, 5.75; N, 19.99. Found: C, 68.43; H, 5.87; N, 19.86.

4.1.2.3. 4-((4-(Cyclohexyloxy)-5-methylpyrimidin-2-yl)amino) benzonitrile (1c). Yield 73%; white solid; mp 176.6–177.4 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.27–1.96 (m, 13H, Me + cyclohexyl H_{2-11}), 5.05–5.09 (m, 1H, cyclohexyl H_1), 7.66 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.91 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 8.07 (s, 1H, pyrimidine H_6), 9.86 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 11.73, 23.32, 25.07, 31.15, 73.51, 101.75, 109.05, 117.85, 119.65, 132.86, 145.29, 157.09, 157.52, 166.75; MS(EI) *m*/*z* 308 (M^+); Anal. calcd for $C_{18}H_{20}N_4O$: C, 70.11; H, 6.54; N, 18.17. Found: C, 70.04; H, 6.65; N, 18.11.

4.1.2.4. 4-((4-(Cyclopentyloxy)-5-methylpyrimidin-2-yl)amino) benzonitrile (1d).

Yield 68%; white solid; mp 154.2–155.1 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.61–2.01 (m, 11H, Me + cyclopentyl H_{2-9}), 5.44 (s, 1H, cyclopentyl H_1), 7.68 (d, J = 8.4 Hz, 2H, Ar$H_{2,6}$), 7.94 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 8.07 (s, 1H, pyrimidine H_6), 9.87 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 11.70, 23.44, 32.39, 78.27, 101.72, 109.18, 117.90, 119.69, 132.91, 145.29, 156.90, 157.50, 167.03; MS(EI) *m*/*z* 294 (M^+); Anal. calcd for $C_{17}H_{18}N_4O$: C, 69.37; H, 6.16; N, 19.03. Found: C, 69.33; H, 6.23; N, 18.89.

4.1.2.5. 4-((4-(Cyclohexyloxy)-6-methylpyrimidin-2-yl)amino) benzonitrile (1e).

Yield 59%; white solid; mp 141.8–142.5 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.23–1.99 (m, 10H, cyclohexyl H_{2-11}), 2.27 (s, 3H, Me), 4.96–5.01 (m, 1H, cyclohexyl H_1), 6.20 (s, 1H, pyrimidine H_5), 7.68 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.94 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 9.99 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.37, 23.54, 25.02, 31.30, 73.79, 98.76, 102.12, 118.18, 119.60, 132.89, 145.14, 158.73, 168.22, 169.12; MS(EI) *m*/*z* 308 (M^+); Anal. calcd for $C_{18}H_{20}N_4O$: C, 70.11; H, 6.54; N, 18.17. Found: C, 70.06; H, 6.62; N, 18.08.

4.1.2.6. 4-((4-(Cyclopentyloxy)-6-methylpyrimidin-2-yl)amino) benzonitrile (1f).

Yield 57%; white solid; mp 158.7–159.4 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.59–1.98 (m, 8H, cyclopentyl H_{2-9}), 2.28 (s, 3H, Me), 5.38 (d, J = 2.8 Hz, 1H, cyclopentyl H_1), 6.22(s, 1H, pyrimidine H_5), 7.71 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.96 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 10.01 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.40, 23.47, 32.35, 78.26, 98.84, 102.09, 118.23, 119.66, 132.99, 145.15, 158.74, 168.13, 169.40; MS(EI) *m*/*z* 294 (M^+); Anal. calcd for $C_{17}H_{18}N_4O$: C, 69.37; H, 6.16; N, 19.03. Found: C, 69.30; H, 6.24; N, 18.97.

4.1.2.7. 4-((4-(Cyclohexylamino)pyrimidin-2-yl)amino)benzonitrile (1g).

Yield 51%; white solid; mp 219.0–220.5 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.16–1.98 (m, 10H, cyclohexyl H_{2-11}), 3.82 (brs, 1H, cyclohexyl H_1), 6.01(d, J = 6.0 Hz, 1H, pyrimidine H_5), 7.24 (brs, 1H, NH), 7.63 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.82 (s, 1H, pyrimidine H_6), 7.99 (d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 9.53 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 24.74, 25.39, 32.41, 48.82, 101.22, 117.93, 119.81, 132.73, 145.88, 159.20, 161.71, MS(ESI+) *m*/*z* 294 $(M+H)^+$; Anal. calcd for $C_{17}H_{19}N_5$: C, 69.60; H, 6.53; N, 23.87. Found: C, 69.52; H, 6.67; N, 23.83.

4.1.2.8. 4-((4-(Cyclopentylamino)pyrimidin-2-yl)amino)benzonitrile (1h).

Yield 54%; white solid; mp 203.6–204.9 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.45–1.97 (m, 8H, cyclopentyl H_{2-9}), 4.25 (brs, 1H, cyclopentyl H_1), 6.01(d, J = 6.0 Hz, 1H, pyrimidine H_5), 7.24 (brs, 1H, NH, deuterium-exchanged), 7.64 (d, J = 8.4 Hz, 2H, Ar$H_{2,6}$), 7.83 (s, 1H, pyrimidine H_6), 8.01 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 9.53 (s, 1H, NH, deuterium-exchanged); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.48, 32.37, 51.67, 99.19, 101.19, 117.96, 119.86, 132.78, 145.90, 154.30, 159.22, 162.15; MS(ESI+) *m*/*z* 280 $(M+H)^+$; Anal. calcd for $C_{16}H_{17}N_5$: C, 68.79; H, 6.13; N, 25.0. Found: C, 68.68; H, 6.27; N, 25.03.

4.1.2.9. 4-((4-(Cyclopropylamino)pyrimidin-2-yl)amino)benzonitrile (1i).

Yield 54%; white solid; mp 183.8–185.4 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 0.49–0.76 (m, 4H, cyclopropyl H_{2-5}), 2.62 (m, 1H, cyclopropyl H_1), 6.11 (d, J = 2.4 Hz, 1H, pyrimidine H_5), 7.52–8.07 (m, 6H, NH + Ar$H_{2,3,4,5}$ + pyrimidine H_6), 9.61 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 6.47, 6.56, 23.25, 101.26, 118.02, 119.88, 132.79, 132.88, 145.85, 159.20, 163.84; MS(ESI+) *m*/*z* 253 $(M+H)^+$; Anal. calcd for $C_{14}H_{13}N_5$: C, 66.92; H, 5.21; N, 27.87. Found: C, 66.88; H, 5.32; N, 27.82

4.1.2.10. 4-((4-(Cyclohexylamino)-5-methylpyrimidin-2-yl) amino)benzonitrile (1j).

Yield 56%; light yellow solid; mp 202.1–203.2 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.12–1.92 (m, 13H, Me + cyclohexyl H_{2-11}), 3.94 (m, 1H, cyclohexyl H_1), 6.34 (d, 1H, J = 7.6 Hz, NH), 7.62 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.69 (s, 1H, pyrimidine H_6), 7.96 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 9.46 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 13.34, 25.25, 25.47, 32.38, 49.50, 100.76, 106.05, 117.51, 119.88, 132.72, 146.11, 153.60, 157.79, 160.30; MS(EI) *m*/*z* 307 (M^+); Anal. calcd for $C_{18}H_{21}N_5$: C, 70.33; H; 6.89; N; 22.78. Found: C, 70.14; H, 7.03; N, 22.69.

4.1.2.11. 4-((4-(Cyclopentylamino)-5-methylpyrimidin-2-yl) amino)benzonitrile (1k).

Yield 55%; light yellow solid; mp 195.7–197.4 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.52–1.98 (m, 11H, Me + cyclopentyl H_{2-9}), 4.37–4.42 (m, 1H, cyclopentyl H_1), 6.44 (d, J = 6.8 Hz, 1H, NH), 7.63 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.70 (s, 1H, pyrimidine H_6), 7.97 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 9.46 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 13.37, 23.61, 32.12, 52.10, 100.73, 106.30, 117.54, 119.92, 132.78, 146.11, 153.34, 157.80, 160.86; MS(EI) *m*/*z* 293 (M^+); Anal. calcd for $C_{17}H_{19}N_5$: C, 69.60; H, 6.53; N, 23.87. Found: C, 69.47; H, 6.79; N, 23.76.

4.1.2.12. 4-((4-(Cyclopropylamino)-5-methylpyrimidin-2-yl) amino)benzonitrile (1l).

Yield 51%; white solid; mp 152.4–152.7 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 0.57–0.82 (m, 4H, cyclopropyl H_{2-5}), 1.90 (s, 3H, Me), 2.80–2.82 (m, 1H, cyclopropyl H_1), 6.91 (d, J = 2.0 Hz, 1H, NH), 7.63 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.71 (s, 1H, pyrimidine H_6), 8.10 (d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 9.55 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 6.49, 13.26, 24.08, 100.73, 106.13, 117.66, 119.98, 132.81, 146.16, 153.63, 157.88, 162.26; MS(ESI+) *m*/*z* 266 $(M+H)^+$; Anal. calcd for $C_{15}H_{15}N_5$: C, 67.90; H, 5.70; N, 26.40. Found: C, 67.84; H, 5.85; N, 26.35.

4.1.2.13. 4-((4-(Cyclohexylamino)-6-methylpyrimidin-2-yl) amino)benzonitrile (1m).

Yield 53%; white solid; mp 200.8–201.1 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.14–1.98 (m, 10H, cyclohexyl H_{2-11}), 2.13 (s, 1H, Me), 3.79 (brs, 1H, cyclohexyl H_1), 5.87 (s, 1H, pyrimidine H_5), 7.08 (brs, 1H, NH), 7.62 (d, J = 8.4 Hz, 2H, Ar$H_{2,6}$), 8.00 (d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 9.54 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.35, 24.80, 25.43, 32.54, 48.98, 96.88, 96.95, 101.03, 117.82, 119.86, 132.71, 146.06, 159.05, 162.36; MS(EI)

m/*z* 307 (M^+); Anal. calcd for $C_{18}H_{21}N_5$: C, 70.33; H, 6.89; N, 22.78. Found: C, 70.22; H, 6.97; N, 22.72.

4.1.2.14. 4-((4-(Cyclopentylamino)-6-methylpyrimidin-2-yl) amino)benzonitrile (1n).

Yield 57%; white solid; mp 177.2–178.5 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.44–1.98 (m, 8H, cyclopentyl H_{2-9}), 2.14 (s, 3H, Me), 4.24 (brs, 1H, cyclopentyl H_1), 5.88 (d, J = 6.0 Hz, 1H, pyrimidine H_5), 7.17 (brs, 1H, NH, deuterium-exchanged), 7.63 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 8.01 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 9.54 (s, 1H, NH, deuterium-exchanged); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.40, 23.50, 32.43, 51.86, 101.00, 117.84, 119.91, 132.77, 146.05, 159.02, 162.81; MS(EI) *m*/*z* 293 (M^+); Anal. calcd for $C_{17}H_{19}N_5$: C, 69.60; H, 6.53; N, 23.87. Found: C, 69.52; H, 6.71; N, 23.7.

4.1.2.15. 4-((4-(Cyclopropylamino)-6-methylpyrimidin-2-yl) amino)benzonitrile (1o).

Yield 51%; white solid; mp 152.4–152.7 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 0.47–0.77 (m, 4H, cyclopropyl H_{2-5}), 2.19 (s, 3H, Me), 2.58 (brs, 1H, cyclopropyl H_1), 6.00 (d, J = 2.4 Hz, 1H, NH), 7.40 (s, 1H, pyrimidine H_5), 7.63 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 8.06 (d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 9.54 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 6.63, 23.26, 23.53, 101.08, 117.92, 119.93, 132.78, 146.01, 158.99, 164.41; MS(ESI+) *m*/*z* 266 $(M+H)^+$; Anal. calcd for $C_{15}H_{15}N_5$: C, 67.90; H, 5.70; N, 26.40. Found: C, 67.81; H, 5.88; N, 26.33.

4.1.2.16. 4-((4-(Cyclohexylthio)pyrimidin-2-yl)amino)benzonitrile (1p).

Yield 51%; light yellow solid; mp 207.1–207.8 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.26–2.05 (m, 10H, cyclohexyl H_{2-11}), 3.83 (s, 1H, cyclohexyl H_1), 6.81 (d, J = 5.6 Hz, 1H, pyrimidine H_5), 7.72 (d, J = 8.4 Hz, 2H, Ar$H_{2,6}$), 7.93 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 8.20 (d, J = 5.2 Hz, 1H, pyrimidine H_6), 10.12 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 25.13, 25.51, 32.58, 41.54, 102.54, 110.97, 118.43, 119.54, 132.95, 144.76, 156.20, 158.74, 169.85; MS(EI) *m*/*z* 310 (M^+); Anal. calcd for $C_{17}H_{18}N_4S$: C, 65.78; H, 5.84; N, 18.05; S, 10.33. Found: C, 65.73; H, 5.97; N, 18.02; S, 10.28.

4.1.2.17. 4-((4-(Cyclohexylthio)-5-methylpyrimidin-2-yl)amino) benzonitrile (1q).

Yield 64%; light yellow solid; mp 134.1–135.6 °C; ^{1}H NMR(400 MHz, $CDCl_3$) δ (ppm) 1.25–2.15 (m, 13H, Me + cyclohexyl H_{2-11}), 3.93–3.98 (m, 1H, cyclohexyl H_1), 7.43 (s, 1H, NH), 7.59 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.74 (d, J = 8.4 Hz, 2H, Ar$H_{3,5}$), 7.92 (s, 1H, pyrimidine H_6); ^{13}C NMR(100 MHz, $CDCl_3$) δ (ppm) 14.95, 25.77, 26.46, 33.29, 42.46, 104.13, 118.13, 119.66, 120.39, 133.29, 144.22, 154.68, 157.11, 170.29; MS(EI) *m*/*z* 324 (M^+) Anal. calcd for $C_{18}H_{20}N_4S$: C, 66.63; H, 6.21; N, 17.27; S, 9.88. Found: C, 66.48; H, 6.42; N, 17.18; S 9.82.

4.1.2.18. 4-((4-(Cyclohexylthio)-6-methylpyrimidin-2-yl) amino)benzonitrile (1r).

Yield 66%; light yellow solid; mp 134.1–135.6 °C; ^{1}H NMR(400 MHz, DMSO-d_6) δ (ppm) 1.27–2.05 (m, 10H, cyclohexyl H_{2-11}), 2.28 (s, 3H, Me), 3.83 (d, J = 2.8 Hz, 1H, cyclohexyl H_1), 6.71 (d, J = 5.6 Hz, 1H, pyrimidine H_5), 7.70 (d, J = 8.8 Hz, 2H, Ar$H_{2,6}$), 7.94 (d, J = 8.8 Hz, 2H, Ar$H_{3,5}$), 10.08 (s, 1H, NH); ^{13}C NMR(100 MHz, DMSO-d_6) δ (ppm) 23.24, 25.15, 25.57, 32.69, 41.48, 102.31, 109.88, 118.28, 119.58, 132.92, 144.95, 158.51, 166.00, 169.42; MS(EI) *m*/*z* 324 (M^+); Anal. calcd for $C_{18}H_{20}N_4S$: C, 66.63; H, 6.21; N, 17.27; S, 9.88. Found: C, 66.59; H, 6.30; N, 17.22; S 9.85

4.2. Anti-HIV activity assay

The anti-HIV activity and cytotoxicity of the compounds **1a–r** were evaluated against wild-type HIV-1 strain IIIB, double RT mutant (K103N + Y181C) HIV-1 and HIV-2 strain ROD in MT-4 cell cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method.[18] Briefly, stock solutions (10 x 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 five-fold dilutions of test compounds 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. Virus 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 compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells were centrifuged for 5 min at 1000 rpm (220 g) 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 viability of mock-and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of yellow colored MTT (Acros Organics) 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 (Safire, Tecan), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of tree wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD540) 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}).

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Supplementary data

Supplementary data (the preparation of intermediate **6–9**, ^{1}H NMR, ^{13}C NMR and MS) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.002.

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