DOI: 10.1002/cmdc.201100334

Synthesis and Anti-HIV Activity of Aryl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazones as Potent Non-nucleoside Reverse Transcriptase Inhibitors

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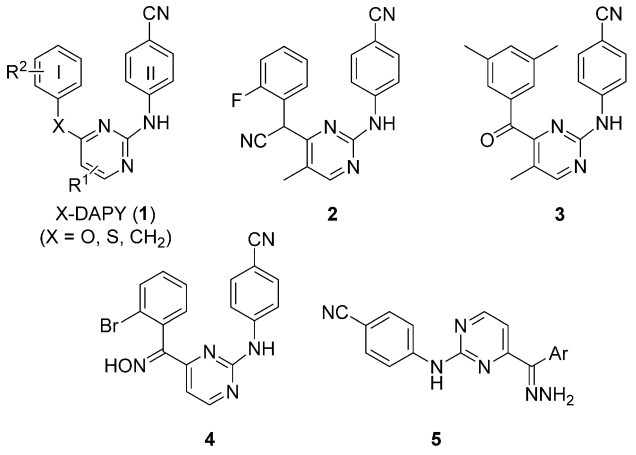
A series of novel diarylpyrimidines (DAPYs) with a ketone hydrazone substituent on the methylene linker between the pyrimidine nucleus and the aryl moiety at the C-4 position were synthesized, and their antiviral activity against human immunodeficiency virus (HIV)-1 in MT-4 cells was evaluated. Most compounds of this class exhibited excellent activity against wild-type HIV-1, with EC_{50} values in the range of 1.7–13.2 nM. Of these compounds, 2-bromophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (**9k**) displayed the most potent anti-HIV-1 activity ($EC_{50} = 1.7 \pm 0.6$ nM), with excellent selectivity for infected over uninfected cells (SI = 5762). In addition, the 4-methyl phenyl analogue **9d** ($EC_{50} = 2.4 \pm 0.2$ nM, SI = 18461) showed broad spectrum HIV inhibitory activity, with EC_{50} values of 2.4 ± 0.2 nM against wild-type HIV-1, 5.3 ± 0.4 μM against HIV-1 double-mutated strain RES056 (K103N + Y181C), and 5.5 μM against HIV-2 ROD strain. Furthermore, structure–activity relationship (SAR) data and molecular modeling results for these compounds are also discussed.

Introduction

Diarylpyrimidine (DAPY) analogues (**1**) represent a class of highly potent non-nucleoside reverse transcriptase inhibitors (NNRTIs), endowed with micromolar to nanomolar activity against wild-type (WT) human immunodeficiency virus (HIV)-1 and clinically relevant mutant HIV-1 strains.[1–5] To identify more potent DAPYs as NNRTIs, we made considerable efforts to chemically modify the methylene linker between the pyrimidine nucleus and phenyl ring I of the diarylpyrimidine, which has given a number of highly active inhibitors, such as cyano-CH_2-DAPYs (**2**),[6] oxo-CH_2-DAPYs (**3**),[7] and oxi-CH_2-DAPYs (**4**).[8]



Encouragingly, the oxi-CH_2-DAPYs were endowed with promising anti-HIV activity with EC_{50} values ranging from 0.569 to 0.005 μM. Therefore, it was of interest to introduce a hydrazine group to the methylene linker, forming hyd-CH_2-DAPYs (**5**), to further improve the hydrogen-bonding and π–π stacking interactions between inhibitors and amino acid residues, Tyr 181 and Tyr 188.[8–11] Herein, a series of aryl 2-[(4-cyanophenyl)amino]-4-pyrimidinyl ketone hydrazones were synthesized, and their anti-HIV activity against WT HIV-1 and HIV-2, as well as against a double-mutated HIV-1 strain (K103N + Y181C) was evaluated. In addition, preliminary structure–activity relationship (SAR) data and molecular modeling results of these new compounds are also discussed.

Results and Discussion

Chemistry

The synthesis of the ketone hydrazones **9a–t** is shown in Scheme 1. The key intermediates, oxo-CH_2-DAPYs (**8a–t**), were conveniently synthesized from 4-(4-chloropyrimidin-2-yl-amino)benzonitriles (**6**) in two steps as previously described.[12,13] Briefly, treatment of **6** with several substituted aryl acetonitriles in the presence of 60% sodium hydride at room temperature for 24–72 h under a N_2 atmosphere afforded cyano-CH_2-DAPYs (**7a–t**). Further oxidation of **7a–t** by bubbling air through the reaction solution at room temperature yielded the oxo-CH_2-DAPYs **8a–t**. Final compounds **9a–t** were synthesized by reac-

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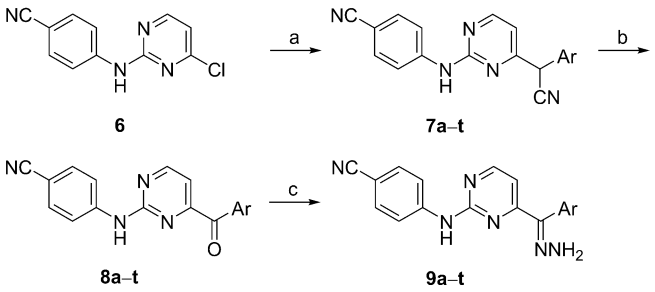


Scheme 1. Synthesis of ketone hydrazone analogues **9a–t**. *Reagents and conditions*: a) Ar-CH_2CN, 60% NaH, DMF, room temperature, 24–72 h, N_2; b) air, DMF, room temperature, 48–72 h; c) NH_2NH_2·2HCl, pyridine, EtOH, reflux, 2 h.

tion of **8a–t** with hydrazine dihydrochloride in the presence of pyridine and ethanol in yields of 28–45%.

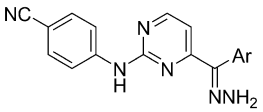
Biological activity

According to the MTT method,[14,15] the anti-HIV activity of compounds **9a–t** was assayed in MT-4 cells infected with WT HIV-1 strain III_B, HIV-1 double-mutant strain RES056 (K103N + Y181C) and HIV-2 strain ROD. Five US Food and Drug Administration (FDA)-approved agents, namely, zidovudine (AZT), nevirapine (NEV), delavirdine (DEV), efavirenz (EFV), and etravirine (ETV), were also included as reference drugs. The results, expressed as CC_{50} (cytotoxicity) and EC_{50} (anti-HIV activity) values, and the calculated selectivity index (SI) given by the CC_{50}/EC_{50} ratio, are listed in Table 1 (HIV-1) and Table 2 (HIV-2).

Most of the compounds showed inhibitory activity against WT HIV-1 (Table 1) with EC_{50} values ranging from 1.7 ± 0.6 to 13.2 ± 5.6 nM, which are superior to those of reference drugs NEV and DEV. In particular, compound **9k** displayed the highest potency with an EC_{50} value of 1.7 ± 0.6 nM against HIV-1, and an SI value of 5762. It is worth noting that several compounds, such as **9d** (EC_{50} = 2.4 ± 0.2 nM, SI = 18461) and **9h** (EC_{50} = 2.6 ± 1.2 nM, SI = 2673), also exhibited the same inhibitory activity as AZT and ETV against WT HIV-1. Additionally, most of the ketone hydrazone derivatives tested proved to be more potent against HIV and less toxic against uninfected cells than the corresponding oxi-CH_2-DAPYs.[8]

In terms of SAR, sterically small substituents, such as methyl (**9d**), chloro (**9h**), and bromo (**9k**) in the *ortho* or *para* position, which would reach the hydrophobic region adjacent to the side chains of Tyr 181 and Tyr 188 of HIV-1 reverse transcriptase (RT), were tolerated well, and these compounds were quite active with EC_{50} values of 2.4 ± 0.2, 2.6 ± 1.2, and 1.7 ± 0.6 nM concentrations, respectively, against HIV-1 III_B-infected MT-4 cells. In the case of di over mono-substitution of phenyl ring I, 2,4-dichloro-substituted **9o** (EC_{50} = 3.8 ± 2.2 nM) did not show a marked improvement on the ability to inhibit WT virus compared with the 2-chloro (**9h**) and 4-chloro (**9j**) analogues (EC_{50} = 2.6 ± 1.2 and 9.1 ± 2.7 nM, respectively), whereas it had a tenfold higher potency than 3,4-substituted analogue **9p** (EC_{50} = 38.0 ± 13.9 nM). In addition, to enhance the π–π stacking interactions between phenyl ring I of the DAPYs and amino acid residues Tyr 181, Tyr 188, and Trp 229 within the binding pocket of RT,[12, 13 16–19] 1-naphthyl- and 2-naphthyl-substituted parent compounds **9s** and **9t** were synthesized and determined to have EC_{50} values of 13.2 ± 5.6 and 8.4 ± 2.1 nM, respectively. It is also noteworthy that 2-naphthyl analogue **9t**

Table 1. Anti-HIV-1 activity and cytotoxicity of compounds **9a–t** in MT-4 cells.[a]



Compd	Ar	EC_{50}[b] [nM] HIV-1 III_B	EC_{50}[b] [μM] K103N + Y181C	CC_{50}[c] [μM]	SI[d]
9a	Ph	11.2 ± 7.0	8.6 ± 1.3	24.8 ± 13.1	2210
9b	2-Me-Ph	6.5 ± 4.2	6.2 ± 2.3	34.7 ± 12.9	5317
9c	3-Me-Ph	10.4 ± 3.3	5.0 ± 0.5	38.0 ± 9.0	3662
9d	4-Me-Ph	2.4 ± 0.2	5.3 ± 0.4	44.2 ± 3.9	18461
9e	2-F-Ph	3.4 ± 1.9	> 7.3	7.3 ± 0.9	2140
9f	3-F-Ph	22.9 ± 10.7	7.5 ± 0.9	21.0 ± 8.3	917
9g	4-F-Ph	39.1 ± 17.8	5.7 ± 0.8	39.5 ± 2.6	1011
9h	2-Cl-Ph	2.6 ± 1.2	> 6.9	6.9 ± 1.0	2673
9i	3-Cl-Ph	19.4 ± 3.6	5.2 ± 0.03	20.7 ± 9.9	1069
9j	4-Cl-Ph	9.1 ± 2.7	12.7 ± 1.3	> 358.4	> 39599
9k	2-Br-Ph	1.7 ± 0.6	≥ 9.2	9.7 ± 4.1	5762
9l	4-Br-Ph	9.6 ± 2.1	> 35.8	35.8 ± 1.8	3725
9m	4-MeO-Ph	3.6 ± 0.8	5.1 ± 0.08	39.4 ± 5.1	10980
9n	4-*t*Bu-Ph	141.4 ± 45.5	> 35.4	35.4 ± 4.5	250
9o	2,4-Cl_2Ph	3.8 ± 2.2	> 10.2	10.2 ± 6.5	2660
9p	3,4-Cl_2Ph	38.0 ± 13.9	≥ 4.3	38.4 ± 3.0	1011
9q	2,5-F_2Ph	9.8 ± 4.2	> 6.3	6.3 ± 1.2	637
9r	3,4-F_2Ph	44.0 ± 2.9	7.1 ± 1.3	144.6 ± 41.0	3288
9s	1-Naphthyl	13.2 ± 5.6	> 42.5	42.5 ± 2.5	3211
9t	2-Naphthyl	8.4 ± 2.1	8.4 ± 3.4	128.8 ± 70.3	15407
AZT[e]	–	6.2 ± 2.1	0.0066 ± 0.0006	> 93.5	> 14988
DEV[e]	–	59.5 ± 7.6	> 4.4	> 4.4	> 74
NEV[e]	–	140.3 ± 58.0	> 15.0	> 15.0	> 107
EFV[e]	–	8.3 ± 4.3	0.49 ± 0.02	> 6.3	> 765
ETV[e]	–	3.0 ± 0.2	0.0256 ± 0.004	> 4.6	> 1537

[a] Data represent the mean ± SD 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} ratio (wild type). [e] Zidovudine (AZT), nevirapine (NEV), delavirdine (DEV), efavirenz (EFV), and etravirine (ETV).

($EC_{50}=8.4\pm2.1$ nM, SI = 15407) showed a slightly higher activity and sevenfold lower cytotoxicity than the corresponding phenyl analogue **9a**, making it a potential lead for further modifications.

The activity of compounds **9a–t** against the known NNRTI-resistant HIV-1 double-mutated (K103N + Y181C) strain RES056 was also determined (Table 1). In this assay, the majority of the tested analogues were still rather active against this virus strain within the 5.0 to 10 μM concentration range. Although less active than reference drugs AZT, EFV, and ETV, almost all of these derivatives displayed higher potency then NEV, and compounds **9c** ($EC_{50}=5.0\pm0.5$ μM), **9d** ($EC_{50}=5.3\pm0.4$ μM), **9i** ($EC_{50}=5.2\pm0.03$ μM), and **9m** ($EC_{50}=5.1\pm0.08$ μM) were nearly as potent as DEV against the double-mutant strain.

Furthermore, the bioactivity of the synthesized compounds was also tested against HIV-2 (strain ROD) in MT-4 cells (Table 2). Interestingly, most of the derivatives, such as **9b** ($EC_{50}=5.8\pm0.02$ μM), **9c** ($EC_{50}=4.9\pm0.2$ μM), **9d** ($EC_{50}=$ 5.5 μM), and **9f** ($EC_{50}=5.8\pm0.1$ μM), exhibited moderate activity at micromolar concentrations, indicating that the ketone hydrazone group on the methylene linker between the pyrimidine nucleus and phenyl ring I of the CH_2-DAPYs did enhance interactions of the inhibitors with their target, HIV-2 reverse transcriptase (RT). Generally, NNRTIs only inhibit HIV-1 replication; however, encouraged by the potency of these analogues against HIV-2 ROD, it was promising to discover a new series of NNRTIs, which targets both HIV-1 and HIV-2 RT, using hyd-CH_2-DAPY as a lead compound.

Table 2. Anti-HIV-2 ROD activity and cytotoxicity of compounds **9a–t** in MT-4 cells.[a]

Compd	EC_{50} [μM][b]	CC_{50} [μM][c]	SI[d]
9a	7.2 ± 0.5	24.8 ± 13.1	3.4
9b	5.8 ± 0.02	34.7 ± 12.9	6.0
9c	4.9 ± 0.2	38.0 ± 9.0	7.8
9d	5.5	44.2 ± 3.9	8.0
9e	> 7.3.1	7.3 ± 0.9	< 1.0
9f	5.8 ± 0.1	39.5 ± 2.6	6.8
9g	≥ 6.7	21.0 ± 8.3	≤ 3.1
9h	> 6.7	6.9 ± 1.0	< 1.0
9i	≥ 4.5	20.7 ± 9.8	≤ 4.6
9j	6.2 ± 1.3	> 358.4	> 57.9
9k	> 9.7	9.7 ± 4.1	< 1.0
9l	> 35.8	35.8 ± 1.8	< 1.0
9m	6.7 ± 1.5	39.4 ± 5.1	5.9
9n	> 35.4	> 35.4	< 1.0
9o	> 10.2	10.2 ± 6.3	< 1.0
9p	> 38.4	38.4 ± 3.0	< 1.0
9q	≥ 2.1	6.3 ± 1.2	≤ 3.0
9r	6.8 ± 0.2	144.6 ± 41.0	21.3
9s	> 42.5	42.5 ± 2.5	< 1.0
9t	10.7 ± 5.0	128.8 ± 70.3	12.1
AZT[e]	0.0042 ± 0.0021	> 93.5	> 22341.4
ETV[e]	0.011 ± 0.00078	> 4.6	> 412.4

[a] Data represent the mean ± SD 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 (SI): CC_{50}/EC_{50} ratio. [e] Zidovudine (AZT), and etravirine (ETV).

Although the target of DAPYs is known to be HIV-1 RT,[1–5] compounds **9d** and **9k** were also evaluated for their activity against HIV-1 $RT_{(WT)}$ at a molecular level, using a poly(rA)/oligo$(dT)_{15}$ homopolymer template with the HIV antigen detection ELISA for quantifying expression of HIV-1 $RT_{(WT)}$ in culture medium,[20] and NVP as a reference compound (Table 3). The results showed that the hyd-CH_2-DAPYs inhibit HIV-1 $RT_{(WT)}$ with IC_{50} values of 0.761 and 0.585 μM for **9d** and **9k**, respectively.

Table 3. Anti-HIV-1 $RT_{(WT)}$ activity of compound **9d** and **9k**.

Compd	IC_{50} [μM][a]
9d	0.761
9k	0.585
NVP	6.947

[a] Compound concentration required to inhibit HIV-1 $RT_{(WT)}$ activity by 50%.

Furthermore, the capacity of analogue **9d** to inhibit the CCR5-tropic and CXCR4-tropic virus strains, which exploit CCR5 and CXCR4, respectively, for viral entry due to the prominent roles these receptors play in the fusion process, was performed using the classical model of detecting calcium mobilization based on co-expression of receptor and Gα16.[21–23] As seen in Figure 1, compound **9d**, which showed broad-spectrum anti-HIV activity, does not effectively inhibit either CCR5 or CXCR4-tropic HIV strains at concentrations greater than 10 μM. Reference compounds maraviroc (formerly UK-427857) and T140 were tested for comparison and gave IC_{50} values of 8.56 and 8.78 nM, respectively.

Molecular simulation

To investigate the potential binding mode of the newly synthesized compounds, molecular simulations were performed by docking potent compounds **9e**, **9f**, **9g**, **9k**, **9m**, and **9n** into the non-nucleoside binding site (NNBS) of wild-type HIV-1 RT (PDB code: 1S6Q),[10] considering their structural diversity and potency. Of these compounds, **9e**, **9f**, and **9g** have the same substituents at different positions of phenyl ring I, whereas **9g**, **9m**, and **9n** are substituted by different groups at the same position. In addition, the most potent compound **9k** and the least active compound **9n** were also selected for docking analysis to compare two derivatives with vastly different activities against WT HIV. Additionally, the binding mode of compound **4** in the NNBS, which is the most active oxi-CH_2-DAPY previously reported by us,[8] was conducted for comparison according to the same protocols. As illustrated in detail in the Experimental Section, the simulations were carried out on the basis of literature protocols,[24–26] using the Base-builder and Surflex-Dock programs interfaced with SYBYL-1.2. The default SYBYL-1.2 parameters were used, and only the key steps and important parameters were interpreted according to the reported general protocols of the Tripos molecular modeling packages Sybyl-X.

As shown in Figure 2, both compounds **9k** and **4** are predicted to display a series of well-known interactions with RT:

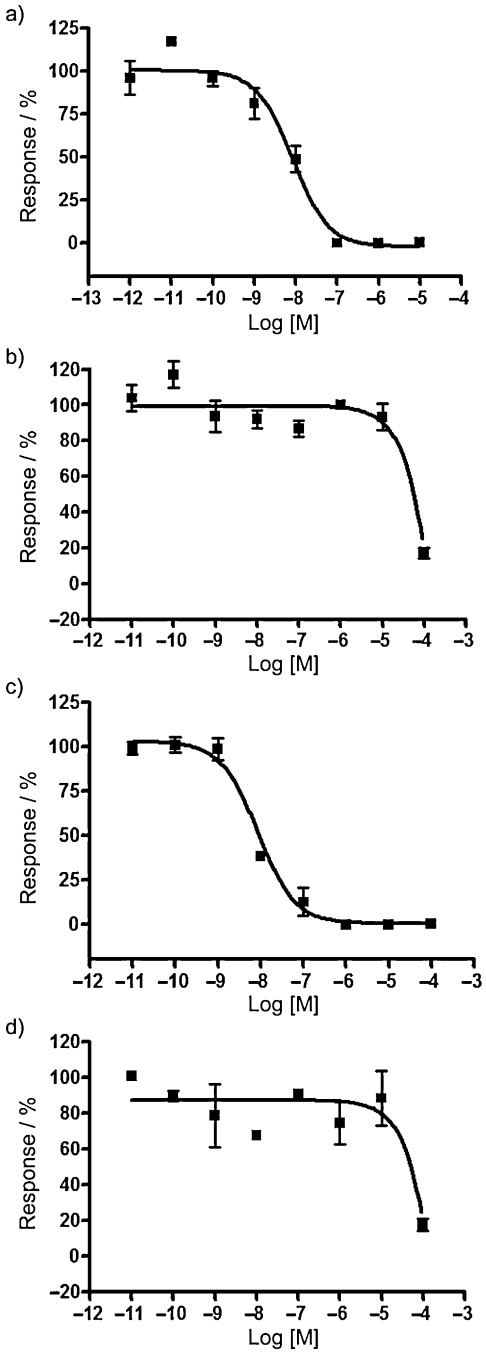


Figure 1. Antagonist models of calcium mobilization assay: a) dose response for maraviroc on CHO/CCR5/Gα16 (regulated on activation, normal T-expressed and secreted (RANTES), 10 nM); b) dose response for **9d** on CHO/CCR5/Gα16 (RANTES, 10 nM); c) dose response for T140 on CHO/CXCR4/Gα16 (SDF-1 30 nM); d) dose response of **9d** on CHO/CXCR4/Gα16 (SDF-1, 30 nM).

a) van der Waals interactions between the cyano group at the C-4 position of phenyl ring II and amino acid residues Leu 100 and Lys 101; b) π–π interactions between phenyl ring I and Tyr 181; c) hydrogen-bonding interactions between the NH group that links the pyrimidine ring and 4-cyanophenyl group with the backbone of Lys 101. As well as these interactions, the NH_2 group of hyd-CH_2-DAPY **9k** is predicted to form another three hydrogen bonds (two between one hydrogen atom of the NH_2 group and the carbonyl of Val 179, and another between the other hydrogen atom of the NH_2 group and the carbonyl of Tyr 188), while the hydroxy group of oxi-CH_2-DAPY (**4**) is predicted to only form two hydrogen bonds (two between the NH_2 group and the carbonyl groups of Val 179 and Tyr 188). This binding model supports our initial design hypothesis that introducing a hydrazine group might improve the hydrogen-bonding interactions of these inhibitors with RT.

To further explore the interactions of newly synthesized compounds with RT, several docking conformations of hyd-CH_2-DAPYs were studied. Similarly to **9k**, compound **9e** had the same interactions with RT, except for the loss of one hydrogen bond between a hydrogen atom of the NH_2 group and the carbonyl of Val 179. Moreover, both compounds, **9f** and **9g** also lost a hydrogen bond with RT (**9f** lost the hydrogen bond with Val 179; **9g** lost the hydrogen bond with Lys 101). In addition, the two least active compounds, **9m** and **9n**, were also docked to the NNBS of HIV-1 RT. The predicted binding modes of compounds **9m** and **9n** are significantly different from that predicted for compound **9k**. Not only are all of the hydrogen bonds with RT predicted to disappear, but these compounds are also predicted to bind out of the NNBS. The predicted poor binding of these compounds with RT is in agreement with the observed significantly weaker anti-HIV activity.

Overall, all these analyses were in accordance with the biological data. Apparently, as designed, the hydrazine group on the methylene linker of hyd-CH_2-DAPY enhanced the interaction with wild-type HIV-1 RT. However, molecular modeling predicts that most of these compounds lack contact with the key mutant amino acid residue Asn 103 in the NNBS, explaining their observed high potency against wild-type HIV-1 but weak activity against the double-mutant strain RES056 (K103N + Y181C).

Conclusions

In summary, led by the oxi-CH_2-DAPYs, a series of new DAPY derivatives with a ketone hydrazine substituent on the methylene linker between the pyrimidine nucleus and phenyl ring I were synthesized and evaluated in cellular assays. Evaluation of their anti-HIV activity indicated that most of the compounds possess antiviral potency superior to that of DEV and NEV against HIV-1 and HIV-2, and equal to that of AZT, EFV, and ETV against HIV-1. In particular, compounds **9d** ($EC_{50} = 2.4 \pm 0.2$ nM, SI = 18461), **9h** ($EC_{50} = 2.6 \pm 1.2$ nM, SI = 2673), and **9k** ($EC_{50} = 1.7 \pm 0.6$ nM, SI = 5762) exhibited more potent activity than ETV against wild-type HIV-1. Furthermore, the activity of compounds **9d** and **9k** against HIV-1 $RT_{(WT)}$, CCR5-tropic HIV, and CXCR4-tropic HIV indicates that the target of hyd-CH_2-DAPYs is wild-type RT only. In addition, compared with the oxi-CH_2-DAPYs, the newly synthesized derivatives exhibit more potent activity and lower cytotoxicity against the chosen HIV strains (HIV-1 III_B, RES056, and HIV-2).

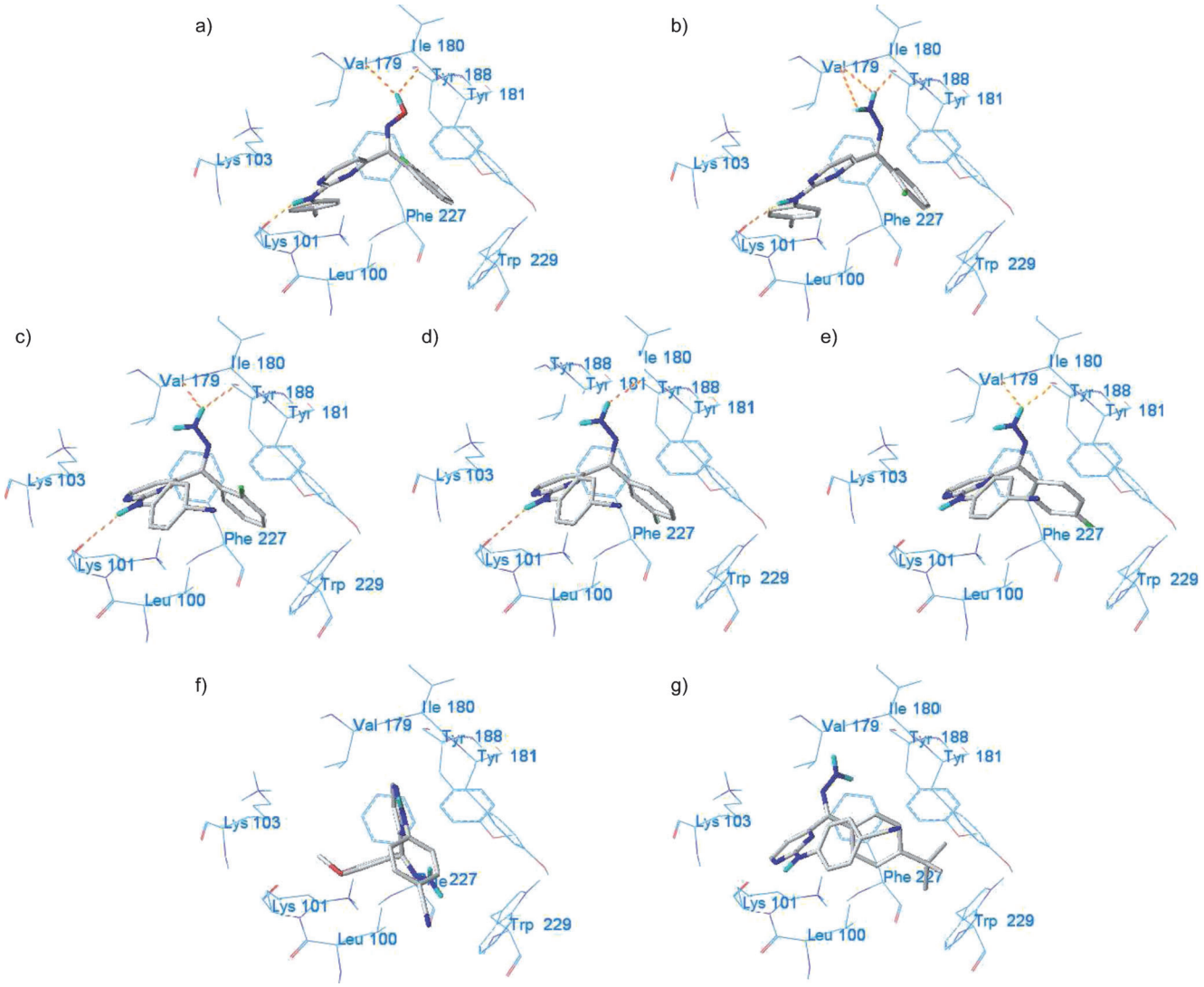


Figure 2. Predicted binding modes and docking poses between the representative compounds and the non-nucleoside binding site (NNBS) of wild-type HIV-1 RT (PDB code: 1S6Q):[10] a) compound **4**; b) compound **9k**; c) compound **9e**; d) compound **9f**; e) compound **9g**; f) compound **9m**; g) compound **9n**. Hydrogen bonds are indicated with dashed lines in red. Hydrogen atoms are omitted for the sake of clarity, except those involved in bonding interactions.

Experimental Section

Chemistry

General: All chemicals used were purchased from commercial sources. Reagents were of analytical grade and used without further purification. Melting points (mp) were measured on a SGW X-1 microscopic melting point apparatus and are uncorrected. Mass spectra (MS) were obtained on a Waters Quattro Micromass instrument using electrospray ionization (ESI). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV400spectrometer in [D$_6$]DMSO. Chemical shifts (δ) are reported in parts per million (ppm) relative to the internal standard, tetramethylsilane (TMS). Elemental analyses were performed on a Carlo Erba 1106 instrument, and the results of elemental analyses for C, H, and N are within $\pm$0.4% of the theoretical values. TLC analyses were run on silica gel 60 F254 plates (Merck) using a variety of solvent systems and a fluorescent indicator for visualization. Spots were visualized under 254 nm UV illumination. Flash chromatography was performed on silica gel (300–400 mesh) using EtOAc and hexane as eluents.

General procedure for the preparation of aryl 2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazones (9a–t): A stirred mixture of **8a–t** (6.0 mmol) and hydrazine dihydrochloride (3.15 g, 30 mmol) in EtOH (30 mL) was treated with pyridine (6.50 mL, 80 mmol) and heated to reflux for 2 h then poured into H_2O (100 mL) and extracted with EtOAc (3×100 mL). The organic layers were combined, washed with 2 N HCl and brine, dried over Na_2SO_4, filtered and concentrated in vacuo to obtain the crude product. The residue was purified by column chromatography on silica gel (20% EtOAc/hexane) to give the desired product as a yellow solid.

4-Methylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9d): Yield: 47%; mp: 183.4–184.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.42 (s, 3H, C*H*$_3$), 7.13–7.62 (m, 8H, Ph*H*), 7.28 (s, 2H, N*H*$_2$), 7.37 (d, 1H, J = 4.8 Hz, C*H*), 8.37 (d, 1H, J = 4.8 Hz, C*H*), 9.90 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 21.0, 101.7, 107.6, 118.1 (2C), 119.6, 129.0, 129.4 (2C), 129.6 (2C), 132.4 (2C), 137.6, 141.0, 145.1, 157.0, 158.9, 161.6 ppm; MS (ESI+): *m/z* (%): 329 [*M*+H]$^+$ (100); Anal. calcd for $C_{19}H_{16}N_6$: C 69.50, H 4.91, N 25.59, found: C 69.53, H 4.87, N 25.60.

Phenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9a): Yield: 43%; mp: 177.8–178.9 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.26–7.61 (m, 9H, Ph*H*), 7.37 (s, 2H, N*H*$_2$), 7.38 (d, 1H, *J* = 5.2 Hz, C*H*), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.93 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.6, 107.5, 118.0 (2C), 119.6, 128.4, 129.0 (2C), 129.5 (2C), 132.1, 132.4 (2C), 140.9, 145.1, 157.1, 158.9, 164.5 ppm; MS (ESI+): *m/z* (%): 315 [*M*+H]$^+$ (100); Anal. calcd for $C_{18}H_{14}N_6$: C 68.78, H 4.49, N 26.74, found: C 68.75, H 4.52, N 26.74.

2-Methylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9b): Yield: 41%; mp: 179.9–180.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.03 (s, 3H, C*H*$_3$), 7.23 (s, 2H, N*H*$_2$), 7.38 (d, 1H, *J* = 5.2 Hz, C*H*), 7.09–7.51 (m, 8H, Ph*H*), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.93 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 19.0, 101.5, 107.2, 117.8 (2C), 119.6, 126.4, 128.6, 129.3, 130.2, 132.2, 132.4, 136.7 (2C), 141.1, 145.1, 157.2, 159.0, 164.3 ppm; MS (ESI+): *m/z* (%): 329 [*M*+H]$^+$ (100); Anal. calcd for $C_{19}H_{16}N_6$: C 69.50, H 4.91, N 25.59, found: C 69.53, H 4.90, N 25.58.

3-Methylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9c): Yield: 44%; mp: 181.1–181.9 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.37 (s, 3H, C*H*$_3$), 7.05–7.61 (m, 8H, Ph*H*), 7.37 (d, 1H, *J* = 5.6 Hz, C*H*), 7.38 (s, 2H, N*H*$_2$), 8.40 (d, 1H, *J* = 5.6 Hz, C*H*), 9.93 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 21.1, 101.7, 107.6, 118.0 (2C), 120.0, 126.5, 129.0, 129.82 (2C), 132.0 (2C), 132.4, 138.2, 141.1, 145.1, 157.1, 158.9, 164.6 ppm; MS (ESI+): *m/z* (%): 329 [*M*+H]$^+$ (100); Anal. calcd for $C_{19}H_{16}N_6$: C 69.50, H 4.91, N 25.59, found: C 69.49, H 4.90, N 25.61.

4-Methylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9d): Yield: 47%; mp: 183.4–184.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.43 (s, 3H, C*H*$_3$), 7.14–7.63 (m, 8H, Ph*H*), 7.29 (s, 2H, N*H*$_2$), 7.36 (d, 1H, *J* = 5.6 Hz, C*H*), 8.38 (d, 1H, *J* = 5.6 Hz, C*H*), 9.91 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 21.0, 101.7, 107.6, 118.1 (2C), 119.6, 129.0, 129.4 (2C), 129.6 (2C), 132.4 (2C), 137.6, 141.0, 145.1, 157.0, 158.9, 164.6 ppm; MS (ESI+): *m/z* (%): 329 [*M*+H]$^+$ (100); Anal. calcd for $C_{19}H_{16}N_6$: C 69.50, H 4.91, N 25.59, found: C 69.53, H 4.87, N 25.60.

2-Fluorolphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9e): Yield: 38%; mp: 209.9–210.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.29–7.65 (m, 8H, Ph*H*), 7.36 (d, 1H, *J* = 5.2 Hz, C*H*), 7.38 (s, 2H, N*H*$_2$), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.94 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.0, 116.2 (J_{CF} = 20.9 Hz), 117.8 (2C), 119.6, 119.7 (J_{CF} = 19.0 Hz), 125.1 (J_{CF} = 3.2 Hz), 131.1 (J_{CF} = 8.5 Hz), 131.6 (J_{CF} = 4.2 Hz), 132.4 (2C), 135.0, 145.1, 157.3, 159.0, 160.1 (J_{CF} = 243.0 Hz), 164.1 ppm; MS (ESI+): *m/z* (%): 333 [*M*+H]$^+$ (100); Anal. calcd for $C_{18}H_{13}FN_6$: C 65.05, H 3.94, N 25.29, F 5.92, found: C 65.04, H 3.95, N 25.32, F 5.91.

3-Fluorolphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9f): Yield: 28%; mp: 196.4–197.5 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.10–7.64 (m, 8H, Ph*H*), 7.37 (d, 1H, *J* = 5.6 Hz, C*H*), 7.51 (s, 2H, N*H*$_2$), 8.40 (d, 1H, *J* = 5.6 Hz, C*H*), 9.94 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.4, 115.2 (J_{CF} = 20.8 Hz), 116.5 (J_{CF} = 124.0 Hz), 118.0 (2C), 119.6, 125.8 (J_{CF} = 2.6 Hz), 131.1 (J_{CF} = 8.3 Hz), 132.4 (2C), 134.6 (J_{CF} = 7.0 Hz), 139.1, 145.1, 157.2, 158.8, 162.7 (J_{CF} = 243 Hz), 164.2 ppm; MS (ESI+): *m/z* (%): 333 [*M*+H]$^+$ (100); Anal. calcd for $C_{18}H_{13}FN_6$: C 65.05, H 3.94, N 25.29, F 5.92, found: C 65.07, H 3.92, N 25.30, F 5.93.

4-Fluorolphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9g): Yield 32%; mp: 194.2–195.4 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.30–7.61 (m, 8H, Ph*H*), 7.32 (d, 1H, *J* = 5.6 Hz, C*H*), 7.43 (s, 2H, N*H*$_2$), 8.39 (d, 1H, *J* = 5.6 Hz, C*H*), 9.92 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.5, 115.9 (2C, J_{CF} = 14.4 Hz), 118.0 (2C), 119.6, 128.4 (J_{CF} = 3.2 Hz), 131.9 (2C, J_{CF} = 8.3 Hz), 132.4 (2C), 139.6, 145.1, 157.1, 158.9, 162.1 (J_{CF} = 242.9 Hz), 164.5 ppm; MS (ESI+): *m/z* (%): 333 [*M*+H]$^+$ (100); Anal. calcd for $C_{18}H_{13}FN_6$: C 65.05, H 3.94, N 25.29, F 5.92, found: C 65.03, H 3.93, N 25.33, F 5.91.

2-Chlorophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9h): Yield: 37%; mp: 195.5–196.4 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.29–7.69 (m, 8H, Ph*H*), 7.38 (d, 1H, *J* = 5.2 Hz, C*H*), 7.51 (s, 2H, N*H*$_2$), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.94 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.6, 106.9, 117.8 (2C), 119.6, 127.9, 129.7, 130.5, 131.6, 131.8, 132.4 (2C), 133.2, 137.9, 145.1, 157.2, 158.9, 163.8 ppm; MS (ESI+): *m/z* (%): 349 [*M*+H]$^+$ (100), 351 [*M*+H+2]$^+$ (32); Anal. calcd for $C_{18}H_{13}ClN_6$: C 61.98, H 3.76, N 24.09, Cl 10.16, found: C 61.99, H 3.75, N 24.11, Cl 10.14.

3-Chlorophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9i): Yield: 35%; mp: 198.4–199.3 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.23–7.62 (m, 8H, Ph*H*), 7.34 (s, 2H, N*H*$_2$), 7.37 (d, 1H, *J* = 5.2 Hz, C*H*), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.95 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.3, 117.9 (2C), 119.6, 128.3 (2C), 129.5, 130.9, 132.4 (2C), 133.7, 134.5, 138.8, 145.1, 157.2, 158.8, 164.2 ppm; MS (ESI+): *m/z* (%): 349 [*M*+H]$^+$ (100), 351 [*M*+H+2]$^+$ (32); Anal. calcd for $C_{18}H_{13}ClN_6$: C 61.98, H 3.76, N 24.09, Cl 10.16, found: C 61.70, H 3.77, N 24.08, Cl 10.13.

4-Chlorophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9j): Yield: 36%; mp: 195.7–196.6 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.27–7.65 (m, 8H, Ph*H*), 7.36 (d, 1H, *J* = 5.2 Hz, C*H*), 7.46 (s, 2H, N*H*$_2$), 8.38 (d, 1H, *J* = 5.2 Hz, C*H*), 9.90 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.4, 118.1 (2C), 119.6, 129.1 (2C), 131.3, 131.7 (2C), 132.4 (2C), 133.1, 139.4, 145.1, 157.2, 158.8, 164.3 ppm; MS (ESI+): *m/z* (%): 349 [*M*+H]$^+$ (100), 351 [*M*+H+2]$^+$ (32); Anal. calcd for $C_{18}H_{13}ClN_6$: C 61.98, H 3.76, N 24.09, Cl 10.16, found: C 61.71, H 3.77, N 24.07, Cl 10.14.

2-Bromophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9k): Yield: 45%; mp: 197.9–198.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.28–7.70 (m, 8H, Ph*H*), 7.51 (s, 2H, N*H*$_2$), 7.38 (d, 1H, *J* = 5.2 Hz, C*H*), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.93 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 102.1, 109.4, 117.8 (2C), 119.5, 122.2, 128.4, 131.0, 131.7, 132.3, 132.5 (2C), 133.0, 144.6, 145.1, 158.7, 159.0, 161.7 ppm; MS (ESI+): *m/z* (%): 393 [*M*+H]$^+$ (100), 395 [*M*+H+2]$^+$ (90); Anal. calcd for $C_{18}H_{13}BrN_6$: C 54.98, H 3.33, Br 20.32, N 21.37, found: C 54.96, H 3.34, Br 20.34, N 21.36.

4-Bromophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9l): Yield: 49%; mp: 199.1–200.3 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.21–7.79 (m, 8H, Ph*H*), 7.36 (d, 1H, *J* = 5.6 Hz, C*H*), 7.52 (s, 2H, N*H*$_2$), 8.39 (d, 1H, *J* = 5.6 Hz, C*H*), 9.92 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.3, 118.0 (2C), 119.6, 121.7 (2C), 131.7, 132.0 (3C), 132.3 (2C), 139.2, 145.0, 157.1, 158.8, 164.3 ppm; MS (ESI+): *m/z* (%): 393 [*M*+H]$^+$ (100), 395 [*M*+H+2]$^+$ (90); Anal. calcd for $C_{18}H_{13}BrN_6$: C 54.98, H 3.33, Br 20.32, N 21.37, found: C 54.97, H 3.34, Br 20.33, N 21.36.

4-Methoxyphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9m): Yield: 32%; mp: 193.9–194.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 3.86 (s, 3H, OC*H*$_3$), 7.13–7.66 (m, 8H, Ph*H*), 7.31 (s, 2H, N*H*$_2$), 7.35 (d, 1H, *J* = 5.2 Hz, C*H*), 8.38 (d, 1H, *J* = 5.2 Hz, C*H*), 9.91 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 55.2, 101.6, 107.7, 114.4 (2C), 118.1 (2C), 119.6, 123.8, 130.9 (2C), 132.4 (2C), 140.8, 145.1, 157.0, 158.9, 159.2, 164.7 ppm; MS (ESI+): *m/z* (%): 345 [*M*+H]$^+$ (100); Anal. calcd for $C_{19}H_{16}N_6O$: C 66.27, H 4.68, N 24.40, found: C 66.28, H 4.67, N 24.41.

4-*t*Butylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9n): Yield: 32%; mp: 179.0–180.1 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.38 (s, 9H, 3C*H*$_3$), 7.20–7.63 (m, 8H, Ph*H*), 7.27 (s, 2H, N*H*$_2$), 7.37 (d, 1H, *J* = 5.2 Hz, C*H*), 8.39 (d, 1H, *J* = 5.2 Hz, C*H*), 9.93 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 31.1 (3C), 34.5, 101.5, 107.7, 118.1 (2C), 119.4, 125.7 (2C), 128.9, 129.1 (2C), 132.3 (2C), 140.8, 145.0, 150.5, 157.1, 158.9, 164.6 ppm; MS (ESI+): *m/z* (%): 371 $[M+H]^+$ (100); Anal. calcd for $C_{22}H_{22}N_6$: C 71.33, H 5.99, N 22.69, found: C 71.31, H 5.60, N 22.70.

2,4-Dichlorophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9o): Yield: 42%; mp: 172.4–173.5 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.31–7.84 (m, 7H, Ph*H*), 7.38 (d, 1H, *J* = 5.2 Hz, C*H*), 7.52 (s, 2H, N*H*$_2$), 8.39 (d, 1H, *J* = 5.2 Hz, C*H*), 9.94 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.8, 106.9, 117.9 (2C), 119.2, 128.3, 129.8, 131.3, 132.8 (2C), 133.2, 134.7, 136.4, 144.4, 145.1, 157.2, 159.2, 163.6 ppm; MS (ESI+): *m/z* (%): 383 $[M+H]^+$ (100), 385 $[M+H+2]^+$ (64); Anal. calcd for $C_{18}H_{12}Cl_2N_6$: C 56.41, H 3.16, Cl 18.50, N 21.93, found: C 56.40, H 3.17, Cl 18.48, N 21.95.

3,4-Dichlorophenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9p): Yield: 40%; mp: 166.7–167.8 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.25–7.84 (m, 7H, Ph*H*), 7.35 (d, 1H, *J* = 5.2 Hz, C*H*), 7.68 (s, 2H, N*H*$_2$), 8.39 (d, 1H, *J* = 5.2 Hz, C*H*), 9.93 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.7, 107.2, 118.0 (2C), 119.5, 130.3, 131.0, 131.2, 131.8, 131.9 (2C), 132.3, 133.4, 137.5, 145.1, 157.1, 158.8, 164.0 ppm; MS (ESI+): *m/z* (%): 383 $[M+H]^+$ (100), 385 $[M+H+2]^+$ (64); Anal. calcd for $C_{18}H_{12}Cl_2N_6$: C 56.41, H 3.16, Cl 18.50, N 21.93, found: C 56.39, H 3.18, Cl 18.49, N 21.94.

2,5-Difluorolphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9q): Yield: 45%; mp: 205.1–205.9 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.20–7.58 (m, 7H, Ph*H*), 7.82 (s, 2H, N*H*$_2$), 7.36 (d, 1H, *J* = 5.2 Hz, C*H*), 8.40 (d, 1H, *J* = 5.2 Hz, C*H*), 9.97 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.8, 106.8, 117.3–117.7 (m, 2C), 117.8 (2C), 117.9–118.2 (m, 1C), 119.6, 121.4–121.7 (m, 1C), 132.4 (2C), 133.2, 145.1, 154.8–157.4 (m, 2C), 157.3, 159.0, 163.8 ppm; MS (ESI+): *m/z* (%): 351 $[M+H]^+$ (100); Anal. calcd for $C_{18}H_{12}F_2N_6$: C 61.71, H 3.45, F 10.85, N 23.99, found: C 61.68, H 3.47, F 10.88, N 23.97.

3,4-Difluorolphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9r): Yield: 42%; mp: 199.9–200.8 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.13–7.66 (m, 7H, Ph*H*), 7.35 (d, 1H, *J* = 5.2 Hz, C*H*), 7.64 (s, 2H, N*H*$_2$), 8.39 (d, 1H, *J* = 5.2 Hz, C*H*), 9.92 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.8, 107.3, 118.0 (2C), 118.2–119.6 (m, 1C), 123.9 (m, 1C), 127.0 (m, 1C), 130.0 (m, 1C), 129.8, 132.3 (2C), 138.0, 145.1, 145.7–161.7 (m, 2C), 157.0, 158.8, 164.1 ppm; MS (ESI+): *m/z* (%): 351 $[M+H]^+$ (100); Anal. calcd for $C_{18}H_{12}F_2N_6$: C 61.71, H 3.45, F 10.85, N 23.99, found: C 61.69, H 3.47, F 10.86, N 23.98.

1-Naphthylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9s): Yield: 31%; mp: 218.9–219.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.21–7.79 (m, 11H, Ph*H*, Naph*H*), 7.52 (s, 2H, N*H*$_2$), 7.36 (d, 1H, *J* = 5.6 Hz, C*H*), 8.39 (d, 1H, *J* = 5.6 Hz, C*H*), 9.92 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.4, 107.2, 117.6 (2C), 119.6, 124.9, 126.2 (2C), 126.6, 127.8, 128.5, 128.7, 130.7 (2C), 132.1 (2C), 133.7, 139.3, 144.9, 157.2, 158.9, 164.8 ppm; MS (ESI+): *m/z* (%): 365 $[M+H]^+$ (100); Anal. calcd for $C_{22}H_{16}N_6$: C 72.51, H 4.43, N 23.06, found: C 72.52, H 4.42, N 23.07.

2-Naphthylphenyl-2-[(4-cyanophenyl)amino]-4-pyrimidinone hydrazone (9t): Yield: 36%; mp: 199.8–200.7 °C; ^{1}H NMR(400 MHz, [D$_6$]DMSO): δ = 6.88–8.13 (m, 11H, Ph*H*, Naph*H*), 7.51 (s, 2H, N*H*$_2$), 7.43 (d, 1H, *J* = 5.2 Hz, C*H*), 8.42 (d, 1H, *J* = 5.2 Hz, C*H*), 9.88 ppm (s, 1H, N*H*); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 101.4, 107.5, 117.9 (2C), 119.5, 126.2, 126.5, 127.2, 127.7, 128.2, 128.5, 129.0, 130.3, 132.1, 132.8 (2C), 133.3, 140.6, 145.0, 157.2, 158.8, 164.7 ppm; MS (ESI+): *m/z* (%): 365 $[M+H]^+$ (100); Anal. calcd for $C_{22}H_{16}N_6$: C 72.51, H 4.43, N 23.06, found: C 72.50, H 4.44, N 23.05.

Biology

Anti-HIV assays: Anti-HIV activity (wild-type HIV-1 strain III$_B$) and cytotoxicity were evaluated in MT-4 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[14,15] Briefly, virus stocks were titrated in MT-4 cells and expressed as the 50% cell culture infective dose ($CCID_{50}$). MT-4 cells were suspended in culture medium at 1×105 cells mL^{-1} and infected with HIV at a multiplicity of infection value of 0.02. Immediately after viral infection, 100 μL of the cell suspension was placed in each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. Stock solutions of the test compounds were dissolved in DMSO at 50 mM or higher. After incubation for four days at 37 °C, the number of viable cells was determined using the MTT method. Compounds were tested in parallel for cytotoxic effects in uninfected MT-4 cells.

RT inhibition assay: According to the method reported by Liu et al.,[20] assays against HIV-1 $RT_{(WT)}$ were performed using a poly(rA)/oligo(dT)$_{15}$ homopolymer template with HIV antigen detection ELISA for quantifying expression of HIV-1 RT in culture medium. Oligo(dT) was immobilized through its 50 terminal phosphate to Covalink-NH microtiter plates. The biotin-11-2′-deoxyuridin-5′-triphosphate (biotin-11-dUTP) was incorporated by wild-type RT. The reaction mixture contained 50 mM Tris·HCl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl, 5 mM 1,4-dithio-D-threitol (DTT), 0.13 mg mL^{-1} bovine serum albumin (BSA), 10 mg mL^{-1} poly(A), 0.75 mM biotin-11-dUTP, and 1.5 mM deoxythymidine triphosphate (dTTP). After incubation at 37 °C for 1 h, the plate was washed three times with a wash buffer containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05 mM $MgCl_2$, and 0.02% Tween-20. After addition of 1% BSA (100 mL), to each well and incubation for 30 min at room temperature, the plate was washed with the same buffer. Subsequently, 50 mL of alkaline phosphatase streptavidin (SA-ALP) solution (100 ng mL^{-1}) was added per well, and the plates were then incubated for 1 h at 37 °C. The plate was washed as described above, and then 50 mL of *p*-nitrophenyl phosphate disodium (PNPP) (1 mg mL^{-1}, pH 9.5) was added. After 30 min at 37 °C, the reaction was stopped by addition of 0.5 M NaOH. The products were detected and quantified using a colorimetric streptavidin alkaline phosphatase reporter system.

Molecular simulation

Molecular modeling was carried out with the Tripos molecular modeling packages Sybyl-1.2. All the molecules for docking were built using standard bond lengths and angles from Sybyl-1.2/base Builder and were then optimized using the Tripos force field for 2000 generations two times or more, until the minimized conformers of the ligand were the same. 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.[24–26] 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 (TMC20), and other unnecessary small molecules from the crystal structure of the TMC20–HIV-1 RT complex (PDB code: 1S6Q);[10] 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 (set to 0), the size to expand search grid (set to 8 Å), the maximum number of rotatable bonds per molecule (set to 100), and the maximum number of poses per ligand (set to 20). 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 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 structure optimization was performed for 20000 generations using a genetic algorithm, and 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 represented the binding affinities of ligand with RT, ranged a wide scope of functional classes (10^{-2}–10^{-9}). Therefore, only the highest-scoring 3D structural model of the ligand-bound RT was chosen to define the binding interaction.[27–29]

Acknowledgements

We are grateful to the National Natural Science Foundation of China (grant nos. 30672536, 20872018 and 81172918) and the Katholieke Universiteit Leuven (Belgium) (GOA no. 10/14) for the financial support of this research. We thank Mrs. K. Erven and Mr. K. Uyttersprot (Rega Institute for Medical Research, Katholieke Universiteit Leuven) for excellent technical assistance.

Keywords: antiviral agents · diarylpyrimidines · HIV-1 · NNRTIs · reverse transcriptase · structure–activity relationships

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Received: July 7, 2011
Published online on September 8, 2011

