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Original article

Design, synthesis and anti-HIV evaluation of novel diarylnicotinamide derivatives (DANAs) targeting the entrance channel of the NNRTI binding pocket through structure-guided molecular hybridization

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ABSTRACT

Through a structure-based molecular hybridization approach, a novel series of diarylnicotinamide derivatives (DANAs) targeting the entrance channel of HIV-1 NNRTIs binding pocket (NNIBP) were rationally designed, synthesized and evaluated for their anti-HIV activities in MT-4 cells together with the inhibition against the reverse transcriptase (RT) in an enzymatic assay. Encouragingly, most of the new DANAs were found to be active against wild-type HIV-1 with an EC_{50} in the range of 0.027–4.54 μM. Among them, compound **6b11** (EC_{50} = 0.027 μM, SI > 12518) and **6b5** (EC_{50} = 0.029 μM, SI = 2471) were identified as the most potent inhibitors, which were more potent than the reference drugs nevirapine (EC_{50} = 0.31 μM) and delavirdine (EC_{50} = 0.66 μM). Some DANAs were also active at micromolar concentrations against the K103N + Y181C resistant mutant. Compound **6b11** exhibited the highest enzymatic inhibition activity (IC_{50} = 20 nM), which is equal to that of efavirenz (EC_{50} = 20 nM) and 31 times higher than that of nevirapine (EC_{50} = 0.62 μM). Preliminary structure-activity relationships (SARs) and molecular modeling of these new DANAs have been discussed.

1. Introduction

Acquired immune deficiency syndrome (AIDS), mainly caused by human immunodeficiency virus type-1 (HIV-1) is still a prevalent disease worldwide. The most common and effective treatment of AIDS is the highly active antiretroviral therapy (HAART). Non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are important components of HAART with high antiviral potency, high specificity and low cytotoxicity [1]. Even so, a major problem is that the long-term efficacy of NNRTIs is limited by the rapid emergence of drug-resistant variants of HIV-1. Hence, the development of novel chemical entities with high affinity for the mutated RT has been a very active research field in recent years [2–4].

Among the more than 50 different series of NNRTIs that have been reported so far, diarylpyrimidine (DAPY) derivatives with superior activity profiles have attracted considerable attention over the past few years [5]. Up to now, two DAPY derivatives, etravirine (**TMC125**, ETV) and rilpivirine (**TMC278**, RPV), have been launched in the marketplace by FDA for the treatment of HIV infection in 2008 and 2011 respectively (Fig. 1). Besides, indolylarylsulfone (IAS) derivatives (lead compound **L-737,126** and **7e**) represent another series of new generation NNRTIs with broad spectrum of activity against drug-resistant HIV strains [4,6,7]. With an attempt to improve their anti-HIV-1 activity, most work on the modification of IASs was focused on introducing different substituents into the 2-carboxamide nitrogen [8–12].

Although DAPYs and IASs belong to two structurally different scaffolds, these two types of NNRTIs share a similar pharmacophoric feature and binding conformation. As shown in Fig. 2, a crystallographic overlay of **TMC125** [13] and **7e** [7] within their NNRTIs binding site (NNIBS) revealed that most fragments in their structures can overlap well with each other. The pyrrolidine ring of **7e** and the 2,6-dimethyl-4-cyanophenoxyl group of **TMC125** both participate in hydrophobic interactions with residues Tyr188 and

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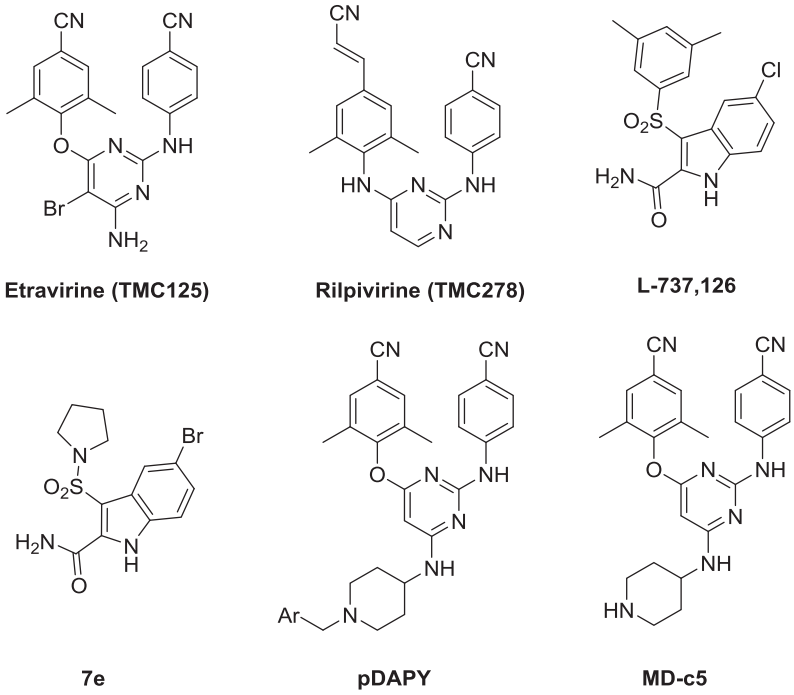


Fig. 1. The structures of representative DAPYs (**TMC125** and **TMC278**), IASs (**L-737,126** and **7e**), **pDAPY** and **MD–c5**.

Tyr188. The phenyl ring of the indolyl group of **7e** can overlap with the right aryl ring of **TMC125**, pointing toward the solvent exposed region. The 2-carbamoyl-pyrrole moiety functioning as the pyrimidine ring of **TMC125** developed critical hydrogen bonding with the backbone of Lys101. In addition, the NH_2 and Br groups on the central pyrimidine ring of **TMC125** pointed to the entrance channel which was surrounded by amino acids Leu100, Lys101, Glu138 and Val179 [13], the substituents at the 2-carboxamide nitrogen may also fit into the entrance channel in the docking study of IAS derivatives [10,14,15].

The entrance channel was first discovered in the structural optimization and molecular modeling study of IAS series [14,15]. More studies suggested that the introduction of nitrogen-containing aromatic heterocycles, amino acids, oligopeptides and so on at the 2-position of the indole ring of IAS will form the double hydrogen bonding with Lys101 and Glu138 of the channel, thus enhancing the potency against the wild-type or mutant virus strains [8–12]. According to the SAR studies of the DAPY derivatives, the substituents on the 5,6-position of the central pyrimidine ring was changeable [16]. The piperidinylamino-diarylpyrimidine (**pDAPY**) derivatives were designed through a molecular hybridization strategy in our previous studies [17]. Among the pDAPY derivatives, compound **MD–c5** was identified as the most potent inhibitor against wild-type and K103N + Y181C drug-resistant mutant HIV-1 strain with an EC_{50} value of 0.038 μM and 0.95 μM respectively (Fig. 1), which confirmed the design rationality of new multi-sites binding NNRTIs especially with the entrance channel targeting property.

Prompted by the analyses of the crystallographic overlay of **TMC125** and **7e**, the novel diarylnicotinamide scaffold was created using crystallographic overlays based molecular hybridization. As shown in Fig. 3, the preferred combination of the trisubstituted phenoxy ring (A-ring), the para-cyanoaniline moiety (B-ring), and the NH group linking the B ring and pyrimidine core (C-ring) of DAPYs were preserved. Substituents at the 2-carboxamide nitrogen in the IASs series were introduced to further improve the interaction with the entrance channel. These newly designed diarylnicotinamide derivatives (DANAs) were synthesized, and their anti-HIV activities against wild-type HIV-1 and HIV-2, as well as their activities against the double mutant HIV-1 strain (K103N + Y181C) were evaluated. Preliminary structure-activity relationships (SARs) and molecular modeling results of these new compounds were also discussed.

2. Chemistry

The expeditious and straightforward synthetic route is depicted in Scheme 1. The synthetic work started with the commercially available diethyl 1,3-acetonedicarboxylate (**1**). Reaction of **1** with triethyl orthoformate in acetic anhydride, followed by cyclisation with ammonia, resulted in formation of ethyl 4,6-dihydroxynicotinate (**2**). Treatment of **2** with phosphorus oxychloride gave ethyl 4,6-dichloronicotinate (**3**), which underwent nucleophilic substitution reaction with different substituted phenols under conditions of DMF/K_2CO_3, generating intermediate ethyl 6-chloro-4-(4-cyano-2,6-dimethylphenoxy)nicotinate (**4a**) and ethyl 6-chloro-4-(mesityloxy)nicotinate (**4b**). Then ethyl 4-(4-cyano-2,6-dimethylphenoxy)-6-(4-cyanophenylamino)nicotinate (**5a**) and ethyl 6-(4-cyanophenylamino)-4-(mesityloxy)nicotinate (**5b**) were obtained from **4a** and **4b** by Buchwald–Hartwig reaction with 4-aminobenzonitrile. The final derivatives **6a1-11** and **6b1-11** were obtained by ammonolysis of **5a** and **5b** using trimethylaluminium [18]. The synthesized compounds were characterized by physicochemical and spectral means. The MS and ^{1}H NMR, ^{13}C NMR spectral data were found in agreement with the assigned molecular structures.

3. Results and discussion

3.1. Anti-HIV activity evaluation

The newly synthesized DANAs were evaluated for their activity against wild-type HIV-1 strain (III_B), K103N + Y181C double mutant HIV-1 strain (RES056) and HIV-2 ROD strain in MT-4 cells using the

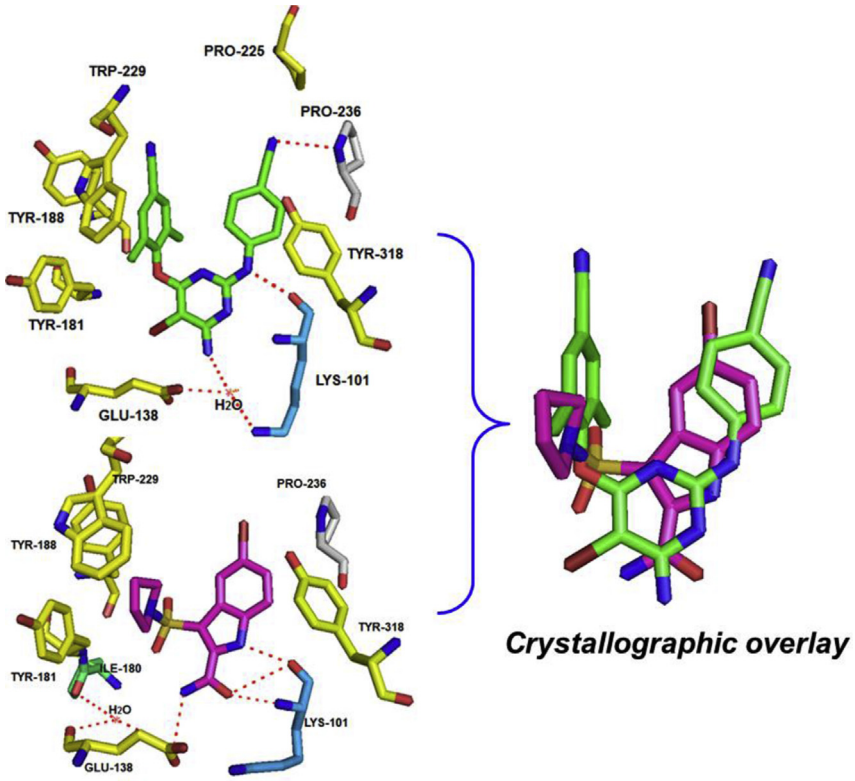


Fig. 2. Crystallographic overlay of the RT co–crystal structures of **TMC125** (green) (PDB code: 3MEC, resolution: 2.3 Å) and **7e** (pink) (PDB code: 2RF2, resolution: 2.4 Å). This figure was generated using PyMOL (www.pymol.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MTT method [19–21]. Nevirapine (NVP), lamivudine (3 TC), zidovudine (azidothymidine, AZT), didanosine (DDI), efavirenz (EFV), delavirdine msylate (DLV) and etravirine (ETV) were used as reference drugs. The cytotoxicity of the compounds was determined in parallel. The results, expressed as EC_{50}, CC_{50} and SI, are illustrated in Table 1.

As shown in Table 1, except for compound **6a1**, all the novel synthesized NNRTIs exhibited moderate (compound **6a6**: EC_{50} = 4.54 μM; **6a8**: EC_{50} = 3.1 μM; **6a3**: EC_{50} = 1.74 μM) to excellent potency (EC_{50} from 0.027 to 0.85 μM) against wild-type HIV-1. Totally, 14 compounds exhibited highly potent inhibitory activity against HIV-1 replication, which were much better than those of NVP, 3 TC, DDI and DLV. Among them, compounds **6b11** (EC_{50} = 0.027 μM, SI > 12,518) and **6b5** (EC_{50} = 0.029 μM, SI = 2471) were the most active derivatives. Especially, **6b11** was about 11 times more active than NVP and 24 times more active than DLV, but still inferior to EFV and ETV. Even so, DANAs merit further modification as potential antiviral agents. Our results unambiguously further validates the molecular hybridization strategy based on crystallographic overlay as an effective strategy for the discovery of novel NNRTIs. None of these compounds showed activity against HIV-2, which means the newly designed DANAs most likely belong to the genuine HIV-1 NNRTIs.

The preliminary SARs analysis indicated that the anti-HIV-1 (III_B) activity is strongly dependent on the nature of the substituents at R_1 and R_2. The detailed discussion of the SAR data for these DANAs will be presented in due course.

Firstly, the methyl group was more favorable to their anti-HIV-1 activities than the nitrile group at the R_1 position which can be clearly observed within **6a** and **6b** series. These results in conjunction with previous studies suggest that the C4-position on the 2,6-dimethylphenyl fragment (A ring) is a major determinant for the anti-HIV-1 activity of DAPY analogues.

Obviously, contrary to our expectations, most of the N-substituted amides resulted in a reduced anti-HIV potency when compared to compound **6a11** or **6b11**. Among the N-substituted amides, the optimum activities were found in compounds with substituents containing a π-electron character. Prop-2-yn-1-yl (**6a5**, **6b5**) appeared to be the most favorable group, closely followed by N-substituents in the sequence prop-2-yn-1-yl > allyl, 2-cyanoethyl. However, bulky substitutions such as 2-(dimethylamino)ethyl (**6a1, 6b1**) or 2,2-dimethoxyethyl (**6a6**, **6b6**) at this position led to significant loss of antiviral potency compared to other analogues in each series. Besides, it is worth noting that the introduction of a hydrophobic ring group may impair the activity, which can be clearly seen from the contrast of compounds **6a4** and **6a2**, **6b4** and **6b2**. In conclusion, SAR investigation revealed that bulky substitutions at the amide position is probably detrimental to activity, while π-electron containing linear substitutions can be tolerated at this position.

Moreover, it cannot be ignored that the replacement of the nitrile in **6a** series by a methyl moiety resulted in **6b** series with significantly exacerbated cytotoxicity (as highlighted by the CC_{50}). In each series, the compounds bearing a 2-(dimethylamino)ethyl group (**6a1** and **6b1**) always exhibited the highest cytotoxicity. It was also noted that the inhibitors containing an unsubstituted amide (**6a11**, **6b11**) revealed a remarkable reduced cytotoxicity with the higher CC_{50} value of >325.95 μM and >335.66 μM, respectively. No wonder the selectivity index of **6a11** and **6b11** were the highest. From what has been discussed above, we can draw the

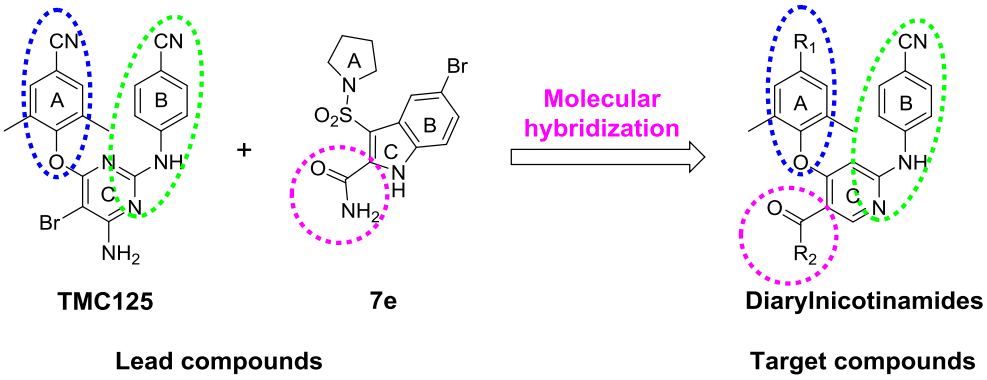


Fig. 3. Design of diarylnicotinamide derivatives (DANAs) based on crystallographic overlay-guided molecular hybridization.

conclusion that the cytotoxicity of DANAs was also directly related to the nature of the substituents on R_1 and R_2 position.

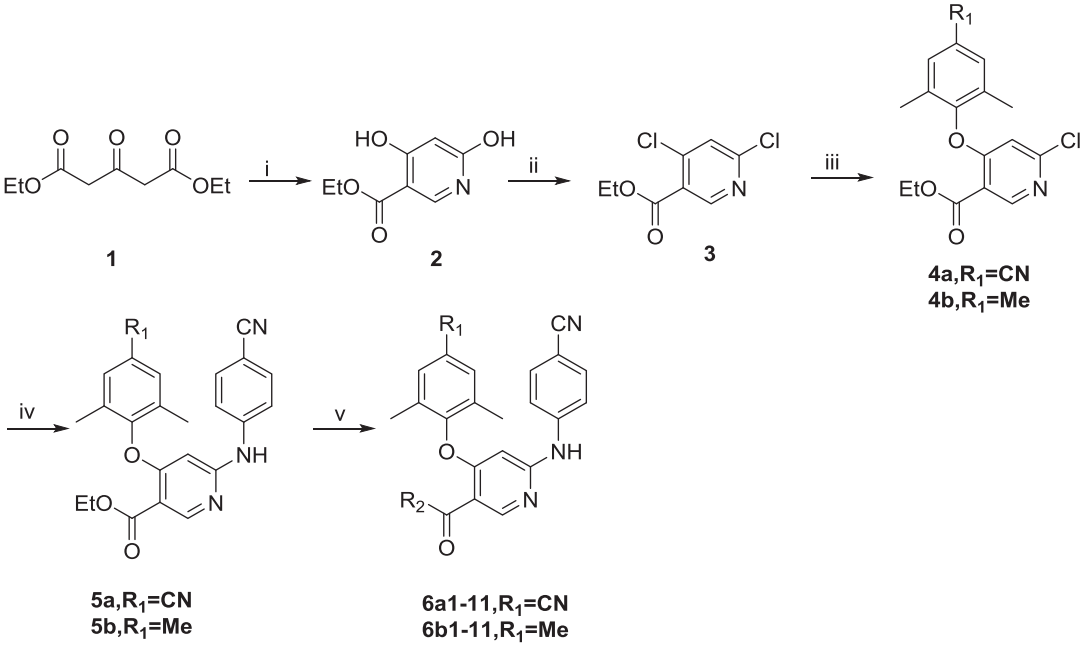
The activity of the compounds against K103N + Y181C double RT mutant strain (RES056), which confers resistance towards most of the classical NNRTIs, was also tested due to the rapid drug resistance emergence in the clinical treatment of AIDS. The results are illustrated in Table 1. Unfortunately, most of the tested compounds were inactive against the RES056 strain except for compounds **6b3**, **6b5** and **6b9**, which showed moderate activities against the RES056 strain with EC_{50} values of 7.8, 6.1 and 10.1 μM respectively. In particular, compound **6b5** was inferior to the reference drugs EFV and ETV, but more potent than the reference drugs NVP and DLV. The results indicated that the existing of π-electron (prop-2-yn-1-yl and allyl) is a key chemical feature responsible for the exceptional biological activities of the compounds against the mutant HIV-1 strain, which can be seen from **6b5** and **6b9**. Even small modifications in the R_2 position of DANAs can impair or restore the activity of the new resulting compounds against the resistant HIV-1 strain, suggesting that these DANAs have the potential to be further optimized as new derivatives with improved antiviral efficacy and favorable drug resistance profiles.

3.2. HIV-1 RT inhibition assay

All the compounds were tested in enzymatic assays against HIV-1 RT (WT) using poly(rA)-oligo(dT) as template/primer and digoxigenin-/biotin-labeled dUTP as nucleotides [22,23] in order to confirm the binding target. Nevirapine (NVP) and efavirenz (EFV) were used as reference drugs. As shown in Table 2, on the whole, the enzymatic activity and SARs of these compounds is in line with the results of the cellular tests. It is reasonable that the newly synthesized compounds can specifically target HIV-1 RT. Among them, eight compounds showed much better RT inhibitory activity than that of NVP. Three compounds (**6a11**, **6b5**, **6b11**) demonstrated extremely high potency compared to that of EFV. Especially, compound **6b11** exhibited the highest enzymatic inhibition activity (IC_{50} = 20 nM), which is equal to that of EFV (EC_{50} = 20 nM) and 31 times higher than that of NVP (EC_{50} = 0.62 μM).

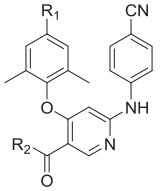
3.3. Molecular modeling analysis

By means of SYBYL-X 1.1 (Tripos, St. Louis, MO, USA), the most potent compound **6b11** was docked into the NNRTIs binding pocket



Scheme 1. Reagents and conditions: i: (a) Acetic anhydride, triethyl orthoformate, 120 °C; (b) NH_3H_2O, rt; ii: $POCl_3$, CH_3CN, triethylamine, 80 °C iii: ArOH, K_2CO_3, DMF, 60 °C; iv: 4-aminobenzonitrile, $Pd(OAc)_2$, Xantphos, Cs_2CO_3, 90 °C; v: Amine, trimethylaluminium, toluene, 80 °C.

Table 1
Activity and cytotoxicity against HIV-1 (III_B and RES056) and HIV-2 (ROD) strains in MT-4 cells.



6a1-11, R1 = CN
6b1-11, R1 = Me

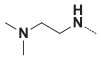
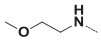
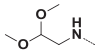
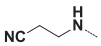
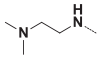
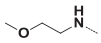
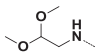
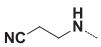
Compds	R_1	R_2	$EC_{50}(\mu M)$[a]			$CC_{50}(\mu M)$[b]	$SI(III_B)$[c]
			III_B	ROD	RES056		
6a1	CN		≥8.8	≥7.5	>23.2	23.19 ± 0.97	≤3
6a2	CN		0.54 ± 0.05	>169.7	>169.7	169.68 ± 27.95	321
6a3	CN		1.74 ± 0.25	>61.0	>61.0	61.04 ± 5.49	35
6a4	CN		0.85 ± 0.13	>146.5	>146.5	≥146.29	≥175
6a5	CN		0.24 ± 0.03	>166.7	>166.7	166.67 ± 13.02	703
6a6	CN		4.54 ± 0.57	>90.6	>90.6	90.56 ± 25.43	20
6a7	CN		0.31 ± 0.06	>231.4	>231.4	231.40 ± 23.50	784
6a8	CN		3.10 ± 0.2	>141.8	>142.5	142.52 ± 22.63	46
6a9	CN		0.24 ± 0.06	>207.1	>207.1	207.08 ± 25.79	625
6a10	CN		0.69 ± 0.06	>65.8	>65.8	65.82 ± 6.6	97
6a11	CN		0.05 ± 0.013	>325.9	>325.9	>325.95	>6195
6b1	Me		0.63 ± 0.12	>16.7	>16.69	4.67 ± 0.37	7
6b2	Me		0.055 ± 0.002	≥11.1	≥11.15	18.89 ± 3.84	335
6b3	Me		0.067 ± 0.006	>24.0	7.8 ± 0.76	24.04 ± 2.00	359
6b4	Me		0.11 ± 0.02	>30.6	>30.57	30.57 ± 10.48	264
6b5	Me		0.029 ± 0.005	>73.0	6.1 ± 0.54	72.96 ± 26.85	2471
6b6	Me		0.28 ± 0.04	>23.2	>23.19	23.19 ± 2.96	83
6b7	Me		0.24 ± 0.02	>25.7	>25.75	25.75 ± 1.77	103
6b8	Me		0.18 ± 0.049	>273.8	>273.82	>273.8	>1492
6b9	Me		0.058 ± 0.002	≥21.5	10.1 ± 0.26	21.33 ± 4.20	364
6b10	Me		0.033 ± 0.002	>9.9	>9.89	9.89 ± 1.59	310
6b11	Me		0.027 ± 0.014	>335.7	>335.66	>335.66	>12,518

Table 1 (*continued*)

Compds	R_1	R_2	EC_{50}(μM)[a]			CC_{50}(μM)[b]	$SI(III_B)$[c]
			III_B	ROD	RES056		
NVP			0.31 ± 0.015	–	≥7.58	>15.02	48
3 TC			2.22 ± 0.19	8.81 ± 0.67	–	>87.24	>39
AZT			0.0071 ± 0.0008	0.0067 ± 0.0004	0.010 ± 0.0025	>93.55	>13,144
DDI			23.20 ± 1.79	44.91 ± 4.59	–	>211.66	>9
EFV			0.0063 ± 0.0005	–	0.16 ± 0.005	>6.34	>1014
DLV			0.66 ± 0.28	–	>43.81	>43.81	>67
ETV[d]			0.0041 ± 0.0001	–	0.025 ± 0.001	>4.59	>1127

[a] EC_{50}: concentration of compound required to achieve 50% protection of MT-4 cell cultures against HIV-1-induced cytotoxicity, as determined by the MTT method.
[b] CC_{50}: concentration required to reduce the viability of mock-infected cell cultures by 50%, as determined by the MTT method.
[c] SI: selectivity index, the ratio of CC_{50}/EC_{50}.
[d] ETV: Etravirine. The data were obtained from the same laboratory (Rega Institute for Medical Research, KU Leuven, Belgium).

(PDB code: 3MEC). The theoretical binding mode of **6b11** to the NNIBP is shown in Fig. 4. The left phenyl ring fitted into the important hydrophobic sub-pocket, which is formed by aromatic amino acid residues Tyr181, Tyr188, Phe227 and Trp229, exhibiting π-π interaction with these residues. The right aniline ring extended to the surface of solvent and protein contacting side chain of amino acid Leu234 and Pro236. Three hydrogen bonds were formed with backbone carbonyl and backbone amino of residue Lys101 and Glu138, improving the affinity between RT and inhibitors. The amide group pointed to the entrance channel forming a hydrogen bond with Glu138. Taken together, the docking study was able to support our original intention and is also in accordance with the biological activity results.

4. Conclusion

In summary, a series of novel DANAs targeting the entrance channel of NNIBP were rationally designed by using a crystallographic overlay-guided hybridization strategy, synthesized and evaluated for their bioactivities against HIV-1 (III_B strain and K103N + Y181C double-mutated strains) and HIV-2 (ROD) in MT-4 cells, as well as against HIV-1 RT activity. These efforts culminated in the discovery of compounds **6b11** (EC_{50} = 0.027 μM, SI > 12,518) and **6b5** (EC_{50} = 0.029 μM, SI = 2471), which showed extremely promising activities against WT HIV-1 with EC_{50} values at double-digit nanomolar level, much better than four clinically-used drugs (NVP, 3 TC, DDI and DLV), and **6b5** also possessed higher potency towards the drug-resistant mutant RES056 strain than NVP and DLV. Besides, the enzymatic activity order of these compounds is essentially in line with the results of the cellular tests. Furthermore, the detailed SARs were discussed. The molecular modeling analysis supported our design hypothesis that the DANAs could interact with the entrance channel of the NNIBP. Taking full advantage of the valuable information from SARs analysis, further optimization of this series of compounds aimed at improving drug resistance profiles and exploring the salient features controlling the activity are ongoing in our lab and will be reported in due course.

Table 2
Inhibitory activity against HIV-1 RT (WT).

Compds	IC_{50}(μM)[a]	Compds	IC_{50}(μM)
6a1	25.77 ± 1.385	**6b1**	3.45 ± 0.198
6a2	4.14 ± 0.125	**6b2**	0.32 ± 0.019
6a3	11.27 ± 0.246	**6b3**	0.52 ± 0.025
6a4	4.25 ± 0.076	**6b4**	0.68 ± 0.011
6a5	1.29 ± 0.025	**6b5**	0.07 ± 0.006
6a6	11.37 ± 1.601	**6b6**	1.89 ± 0.291
6a7	3.37 ± 0.358	**6b7**	0.23 ± 0.008
6a8	10.92 ± 0.430	**6b8**	1.20 ± 0.105
6a9	2.85 ± 0.070	**6b9**	0.17 ± 0.016
6a10	3.94 ± 0.214	**6b10**	0.19 ± 0.008
6a11	0.08 ± 0.003	**6b11**	0.02 ± 0
NVP	0.62 ± 0.038	**EFV**	0.02 ± 0.002

[a] IC_{50}: Inhibitory concentration of tested compounds required to inhibit biotin deoxyuridine triphosphate (biotin-dUTP) incorporation into the HIV-1 RT by 50%.

5. Experimental section

5.1. Chemistry

All melting points were determined on a micromelting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet) or m (multiplet). Mass spectra were taken on an LC Autosampler Device: Standard G1313A instrument. TLC was performed on Silica Gel GF254 for TLC (Merck) and spots were visualized by iodine vapors or by irradiation with UV light (254 nm). Flash column chromatography was performed on column packed with Silica Gel 60 (200–300 mesh). Solvents were reagent grade and, when necessary, were purified

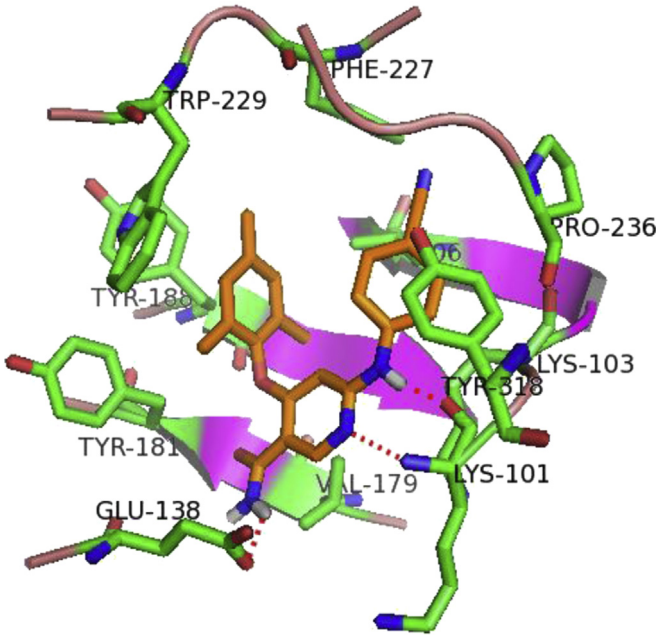


Fig. 4. Predicted binding modes of **6b11** in the NNIBP of wild-type HIV-1 RT (PDB code: 3MEC) displayed by PyMOL.

and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure.

5.1.1. General procedure for the synthesis of ethyl 4,6-dihydroxynicotinate (**2**)

Diethyl 3-oxopentanedioate (**1**) is commercially available. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 4.10 (q, 4H, J = 7.1, 2 × CH_2), 3.69 (s, 4H, 2 × CH_2), 1.20 (t, 6H, J = 7.1, 2 × CH_3), ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 197.34, 167.11 (2 × C), 61.07 (2 × C), 49.23 (2 × C), 14.31 (2 × C).

Diethyl 3-oxopentanedioate (**1**, 10.00 g, 49.45 mmol), triethyl orthoformate (7.33 g, 49.45 mmol) and acetic anhydride (10.10 g, 98.93 mmol) were combined and stirred at 120 °C for 2 h then allowed to cool to ambient temperature. The volatiles were removed under vacuum. The residue was then cooled in an ice bath and aqueous ammonia (25 mL) was added in portions with stirring. The reaction mixture was stirred at 0 °C for 1 h and was then acidified with 2N hydrochloric acid to pH < 5. The precipitate was collected by filtration and allowed to dry to provide **2** as yellow solid. Yield: 81.6%. Mp: 207–210 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 11.78 (s, br, 1H), 10.74 (s, br, 1H), 8.02 (s, 1H), 5.61 (s, 1H), 4.27 (q, 2H, J = 7.1), 1.29 (t, 3H, J = 7.1). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 166.57, 166.40, 164.00, 142.97, 100.44, 98.96, 61.35, 14.46.

5.1.2. General procedure for the synthesis of ethyl 4,6-dichloronicotinate (**3**)

The reaction mixture of ethyl 4,6-dihydroxynicotinate (**2**, 5.6 g, 30.57 mmol), phosphorus oxychloride (23.44 g, 152.87 mmol) and triethylamine (7.73 g, 76.39 mmol) in 40 mL CH_3CN was stirred at 80 °C for 5 h. After removal of the solvent under reduced pressure, water (20 mL) was added at 0 °C. The reaction mixture was alkalized with K_2CO_3 to pH > 7 and extracted with ethyl acetate (3 × 10 mL). The combined organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by silica gel using ethyl acetate-petroleum ether. Pure fractions were collected and concentrated, giving the desired compounds **3** as colorless oil. Yield: 85.1%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 8.83 (s, 1H), 7.97 (s, 1H), 4.38 (q, 2H, J = 7.1), 1.35 (t, 3H, J = 7.1). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.12, 153.97, 152.15, 145.00, 126.30, 125.77, 62.45, 14.36. ESI-MS: m/z 220.2 (M + 1), 222.2 (M + 3). $C_8H_7Cl_2NO_2$ (218.99).

5.1.3. General procedure for the synthesis of ethyl 6-chloro-4-(4-cyano-2,6-dimethylphenoxy)nicotinate (**4a**) and ethyl 6-chloro-4-(mesityloxy)nicotinate (**4b**)

The reaction mixture of 4-hydroxy-3,5-dimethylbenzonitrile (1.12 g, 7.61 mmol) and K_2CO_3 (2.63 g, 19.06 mmol) in 30 mL DMF was stirred at ambient temperature for 15 min. Ethyl 4,6-dichloronicotinate (**3**, 2.0 g, 9.09 mmol) was added and the resulting mixture was stirred at 50 °C for 24 h. After removal of the solvent under reduced pressure, water (20 mL) was added and extracted with ethyl acetate (3 × 10 mL). The combined organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by silica gel using ethyl acetate-petroleum ether. Pure fractions were collected and concentrated, giving the desired compounds **4a** as white crystals. Yield: 50.1%. Mp: 95–100 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 8.78 (s, 1H, pyridine-H), 7.78 (s, 2H, PhH), 6.58 (s, 1H, pyridine-H), 4.37 (q, 2H, J = 7.1, CH_2), 2.12 (s, 6H, 2 × CH_3), 1.33 (t, 3H, J = 7.1, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.32, 163.29, 155.60, 153.13, 152.88, 133.99 (2 × C), 132.71 (2 × C), 118.82, 116.46, 109.93, 109.49, 61.92, 15.78 (2 × C), 14.47. ESI-MS: m/z 331.4 (M + 1), 333.5 (M + 3). $C_{17}H_{15}ClN_2O_3$ (330.08).

4b was synthesized as for **4a** but using 2,4,6-trimethylphenol. White crystals, yield: 46.4%. Mp: 74–77 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 8.74 (s, 1H, pyridine-H), 7.04 (s, 2H, PhH), 6.36 (s, 1H, pyridine-H), 4.37 (q, 2H, J = 7.1, CH_2), 2.29 (s, 3H, CH_3), 2.03 (s, 6H, 2 × CH_3), 1.33 (t, 3H, J = 7.1, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 164.50, 163.52, 155.30, 153.03, 147.10, 136.22, 130.46 (2 × C), 129.94, 116.52, 109.02, 61.80, 20.83, 15.85 (2 × C), 14.47. ESI-MS: m/z 320.3 (M + 1), 322.4 (M + 3). $C_{17}H_{18}ClNO_3$ (319.10).

5.1.4. General procedure for the synthesis of ethyl 4-(4-cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl)amino)nicotinate (**5a**) and ethyl 6-((4-cyanophenyl)amino)-4-(mesityloxy)nicotinate (**5b**)

Palladium acetate (0.01543 g, 0.069 mmol) and Xantphos (0.0796 g, 0.138 mmol) were dissolved in 15 mL dioxane and stirred at ambient temperature for 15 min. 4-aminobenzonitrile (0.1625 g, 1.38 mmol), **4a** (0.50 g, 1.52 mmol) and Cs_2CO_3 (0.673 g, 2.07 mmol) were added, and the flask was evacuated and backfilled with nitrogen for three times. The resulting mixture was stirred at 90 °C for 12 h. The mixture was filtrated and the filtrate was collected and concentrated. The crude residue was purified by silica gel chromatography to provide the title product **5a** as white crystals. Yield: 77%. Mp: 243–245 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.81 (s, 1H, NH), 8.72 (s, 1H, pyridine-H), 7.84 (d, 2H, J = 7.3 PhH), 7.83 (s, 2H, PhH), 7.71 (d, 2H, J = 8.8, PhH), 5.88 (s, 1H, pyridine-H), 4.32 (q, 2H, J = 7.1, CH_2), 2.15 (s, 6H, 2 × CH_3), 1.32 (t, 3H, J = 7.1, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.89, 162.88, 159.39, 153.60, 153.37, 145.15, 133.79, 133.61, 133.09, 119.91, 118.85, 118.79, 109.43, 109.26, 103.08, 94.97, 60.90, 15.83 (2 × C), 14.63. ESI-MS: m/z 413.5 (M + 1), 435.5 (M + 23). $C_{24}H_{20}N_4O_3$ (412.15).

5b was synthesized as for **5a** but using **4b**.White powder, yield: 53.8%. Mp: 243–245 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.83 (s, 1H, NH), 8.69 (s, 1H, pyridine-H), 7.86 (d, 2H, J = 7.1, PhH), 7.69 (d, 2H, J = 8.0, PhH), 7.04 (s, 2H, PhH), 5.94 (s, 1H, pyridine-H), 4.32 (q, 2H, J = 7.1, CH_2), 2.30 (s, 3H, CH_3), 2.21 (s, 6H, 2 × CH_3), 1.32 (t, 3H, J = 7.1, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 164.16, 164.11, 159.39, 153.04, 147.62, 145.41, 135.53, 133.51, 130.28, 130.26, 119.96, 118.73, 109.52, 102.84, 94.88, 60.73, 20.82, 16.01 (2 × C), 14.63. ESI-MS: m/z 402.5 (M + 1), 424.5 (M + 23). $C_{24}H_{23}N_3O_3$ (401.17).

5.1.5. General procedure for the synthesis of **6a1-11** and **6b1-11**

Trimethylaluminium (1.6 M in toluene) (1.75 mL, 2.8 mmol) was added dropwise at 0 °C to a solution of different amine (2.8 mmol) in dry toluene (10 mL) under nitrogen atmosphere and the reaction allowed to stir for 1 h. Then **5a** or **5b** (0.70 mmol) was added and the reaction was stirred at 70 °C for 12 h. Water (20 mL) was added and extracted with ethyl acetate (3 × 10 mL). The combined organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by silica gel using ethyl acetate-petroleum ether to provide the title product **6a1-10** and **6b1-10**, **6a11** and **6b11** were obtained by reaction in ammonia gas atmosphere.

5.1.6. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl)amino)-N-(2-(dimethylamino)ethyl)nicotinamide (**6a1**)

White powder, yield: 46.3%. Mp: 238–240 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.69 (s, 1H, NH), 8.70 (s, 1H, pyridine-H), 8.18 (t, 1H, J = 5.2, NH), 7.85 (s, 2H, PhH), 7.84 (d, 2H, J = 9.2, PhH), 7.69 (d, 2H, J = 8.8, PhH), 5.87 (s, 1H, pyridine-H), 3.41 (q, 2H, J = 5.6, CH_2), 2.42 (t, 2H, J = 6.5, CH_2), 2.17 (s, 6H, 2 × CH_3), 2.14 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.06, 161.42, 158.46, 153.21, 152.31, 145.46, 133.75, 133.60, 133.31, 120.00, 118.80, 118.43, 112.36, 109.61, 102.61, 94.43, 58.14, 45.44 (2 × C), 37.57, 15.86 (2 × C). ESI-MS: m/z 455.5 (M + 1). $C_{26}H_{26}N_6O_2$ (454.21).

*5.1.7. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-propylnicotinamide (**6a2**)*

White powder, yield: 48.5%. Mp: 278–281 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.66 (s, 1H, NH), 8.64 (s, 1H, pyridine-H), 8.18 (t, 1H, J = 5.6, NH), 7.84 (d, 2H, J = 8.6, PhH), 7.83 (s, 2H, PhH), 7.68 (d, 2H, J = 8.7, PhH), 5.88 (s, 1H, pyridine-H), 3.33–3.27 (m, 2H, CH_2), 2.18 (s, 6H, 2 × CH_3), 1.56 (q, 2H, J = 7.2, CH_2), 0.90 (t, 3H, J = 7.4, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.34, 161.39, 158.24, 153.42, 151.70, 145.55, 133.70, 133.57, 133.38, 120.00, 118.82, 118.36, 113.33, 109.53, 102.52, 94.56, 41.29, 22.91, 15.94 (2 × C), 11.77. ESI-MS: m/z 426.5 (M + 1), 448.5 (M + 23). $C_{25}H_{23}N_5O_2$ (425.19).

*5.1.8. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-(2-methoxyethyl)nicotinamide (**6a3**)*

White powder, yield: 56.1%. Mp: 242–244 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.69 (s, 1H, NH), 8.69 (s, 1H, pyridine-H), 8.18 (s, 1H, NH), 7.84 (s, 2H, PhH), 7.84 (d, 2H, J = 7.8, PhH), 7.69 (d, 2H, J = 8.8, PhH), 5.88 (s, 1H, pyridine-H), 3.51–3.49 (m, 4H, 2 × CH_2), 3.26 (s, 3H, CH_3), 2.17 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.36, 161.42, 158.45, 153.29, 152.19, 145.47, 133.74, 133.59, 133.38, 119.99, 118.81, 118.43, 112.49, 109.61, 102.62, 94.51, 70.95, 58.41, 39.26, 15.90 (2 × C). ESI-MS: m/z 442.5 (M + 1), 464.4 (M + 23). $C_{25}H_{23}N_5O_3$ (441.18).

*5.1.9. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-(cyclopropylmethyl)nicotinamide (**6a4**)*

White powder, yield: 84.9%. Mp: 258–260 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.68 (s, 1H, NH), 8.66 (s, 1H, pyridine-H), 8.24 (t, 1H, J = 5.7, NH), 7.84 (d, 2H, J = 8.8, PhH), 7.83 (s, 2H, PhH), 7.69 (d, 2H, J = 8.8, PhH), 5.88 (s, 1H, pyridine-H), 3.22 (t, 2H, J = 6.2, CH_2), 2.19 (s, 6H, 2 × CH_3), 1.10–1.06 (m, 1H, CH), 0.43–0.42 (m, 2H, CH_2), 0.26–0.24 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.31, 161.40, 158.30, 153.42, 151.89, 145.53, 133.72, 133.59, 133.39, 120.00, 118.83, 118.38, 113.06, 109.54, 102.54, 94.56, 43.56, 15.96 (2 × C), 11.48, 3.47 (2 × C). ESI-MS: m/z 438.5 (M + 1), 460.5 (M + 23). $C_{26}H_{23}N_5O_2$ (437.19).

*5.1.10. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-(prop-2-yn-1-yl)nicotinamide (**6a5**)*

White powder, yield: 81.6%. Mp: 258–259 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.72 (s, 1H, NH), 8.69 (s, 1H, pyridine-H), 8.58 (t, 1H, J = 5.6, NH), 7.85 (s, 2H, PhH), 7.84 (s, 2H, J = 7.0, PhH), 7.69 (d, 2H, J = 8.8, PhH), 5.88 (s, 1H, pyridine-H), 4.10 (dd, 2H, J_1 = 2.3, J_2 = 5.6, CH_2), 3.10 (t, 1H, J = 2.4, CH), 2.18 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.1, 161.47, 158.60, 153.37, 152.31, 145.42, 133.71, 133.60, 133.42, 119.99, 118.83, 118.49, 112.11, 109.57, 102.69, 94.50, 82.08, 72.93, 29.12, 15.99 (2 × C). ESI-MS: m/z 422.4 (M + 1), 444.5 (M + 23). $C_{25}H_{19}N_5O_2$ (421.15).

*5.1.11. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-(2,2-dimethoxyethyl)nicotinamide (**6a6**)*

White powder, yield: 70.0%. Mp: 246–248 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.70 (s, 1H, NH), 8.69 (s, 1H, pyridine-H), 8.14 (t, 1H, J = 5.7, NH), 7.85 (s, 2H, PhH), 7.84 (d, 2H, J = 8.2, PhH), 7.69 (d, 2H, J = 8.9, PhH), 5.88 (s, 1H, pyridine-H), 4.57 (t, 1H, J = 5.6, CH), 3.45 (t, 2H, J = 5.6, CH_2), 3.30 (s, 6H, 2 × CH_3), 2.18 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.42, 161.45, 158.54, 153.24, 152.27, 145.44, 133.76, 133.59, 133.36, 119.98, 118.79, 118.47, 112.25, 109.65, 102.67, 102.38, 94.49, 53.87 (2 × C), 41.39, 15.87 (2 × C). ESI-MS: m/z 472.4 (M + 1), 494.4 (M + 23). $C_{26}H_{25}N_5O_4$ (471.19).

*5.1.12. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-cyclopropylnicotinamide(**6a7**)*

White powder, yield: 74.0%. Mp: 286–290 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.65 (s, 1H, NH), 8.50 (s, 1H, pyridine-H), 8.18 (d, 1H, J = 4.1, NH), 7.83 (d, 2H, J = 9.2, PhH), 7.82 (s, 2H, PhH), 7.68 (d, 2H, J = 8.8, PhH), 5.86 (s, 1H, pyridine-H), 2.89–2.84 (m, 1H, CH), 2.16 (s, 6H, 2 × CH_3), 0.74–0.70 (m, 2H, CH_2), 0.60–0.59 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 164.97, 161.36, 158.05, 153.51, 150.79, 145.57, 133.69, 133.58, 120.01, 118.84, 118.29, 114.15, 109.44, 102.46, 94.75, 23.41, 15.95 (2 × C), 6.61 (2 × C). ESI-MS: m/z 424.5 (M + 1), 446.4(M + 23). $C_{25}H_{21}N_5O_2$ (423.17).

*5.1.13. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)-N-((tetrahydrofuran-2-yl)methyl)nicotinamide (**6a8**)*

White powder, yield: 74.1%. Mp: 258–259 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.68 (s, 1H, NH), 8.67 (s, 1H, pyridine-H), 8.13 (t, 1H, J = 5.7, NH), 7.84 (s, 2H, PhH), 7.83 (d, J = 8.8, 2H, PhH), 7.68 (d, 2H, J = 8.8, PhH), 5.87 (s, 1H, pyridine-H), 4.00 (q, 1H, J = 6.1, CH), 3.74–3.70 (m, 1H, CH_2), 3.63–3.57 (m, 1H, CH_2), 3.40 (td, 2H, J_1 = 5.8, J_2 = 1.9, CH_2), 2.17 (s, 6H, 2 × CH_3), 1.93–1.87 (m, 1H, CH_2), 1.85–1.76 (m, 2H, CH_2), 1.65–1.58 (m, 1H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.44, 161.39, 158.43, 153.22, 152.12, 145.47, 133.78, 133.60, 133.34, 119.99, 118.79, 118.43, 112.58, 109.66, 94.51, 77.45, 67.71, 43.34, 28.78, 25.70, 15.90. ESI-MS: m/z 468.4 (M + 1), 490.5 (M + 23). $C_{27}H_{25}N_5O_3$ (467.20).

*5.1.14. N-allyl-4-(4-cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl)amino)nicotinamide(**6a9**)*

White powder, yield: 89.6%. Mp: 163–165 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.69 (s, 1H, NH), 8.67 (s, 1H, pyridine-H), 8.36 (t, 1H, J = 5.8, NH), 7.84 (s, 2H, PhH), 7.83 (d, J = 8.8, 2H, PhH), 7.69 (d, 2H, J = 8.8, PhH), 5.98–5.89 (m, 1H, C]CH), 5.88 (s, 1H, pyridine-H), 5.19 (dd, 1H, J_1 = 17.2, J_2 = 1.7, C]CH_2), 5.09 (dd, 1H, J_1 = 10.3, J_2 = 1.6, C]CH_2), 3.97–3.95 (m, 2H, CH_2), 2.18 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.25, 161.43, 158.36, 153.37, 151.92, 145.51, 136.03, 133.71, 133.59, 133.40, 120.00, 118.82, 118.40, 115.08, 112.94, 109.55, 102.57, 94.51, 41.81, 15.97 (2 × C). ESI-MS: m/z 424.5 (M + 1), 446.4 (M + 23). $C_{25}H_{21}N_5O_2$ (423.17).

*5.1.15. 4-(4-Cyano-2,6-dimethylphenoxy)-N-(2-cyanoethyl)-6-((4-cyanophenyl)amino)nicotinamide (**6a10**)*

White powder, yield: 75.6%. Mp: 235–238 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.73 (s, 1H, NH), 8.74 (s, 1H, pyridine-H), 8.54 (t, 1H, J = 5.5, NH), 7.85 (s, 2H, PhH), 7.84 (d, J = 7.5, 2H, PhH), 7.70 (d, 2H, J = 8.8, PhH), 5.89 (s, 1H, pyridine-H), 3.58 (q, 2H, J = 6.1, CH_2), 2.83 (t, 2H, J = 6.4, CH_2), 2.19 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.58, 161.53, 158.70, 153.34, 152.55, 145.40, 133.69, 133.60, 133.49, 119.98, 119.69, 118.83, 118.51, 111.76, 109.59, 102.72, 94.36, 35.79, 18.06, 15.97 (2 × C). ESI-MS: m/z 437.5 (M + 1), 459.5 (M + 23). $C_{25}H_{20}N_6O_2$ (436.16).

*5.1.16. 4-(4-Cyano-2,6-dimethylphenoxy)-6-((4-cyanophenyl) amino)nicotinamide (**6a11**)*

White powder, yield: 57.6%. Mp: >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.68 (s, 1H, NH), 8.71 (s, 1H, pyridine-H), 7.84 (d, 2H, J = 8.4, PhH), 7.83 (s, 2H, PhH), 7.69 (d, 2H, J = 8.8, PhH), 7.61 (s, 2H, NH_2), 5.86 (s, 1H, pyridine-H), 2.17 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 164.82, 161.64, 158.58, 153.36, 152.55, 145.49, 133.70, 133.58, 133.37, 119.99, 118.83, 118.44, 112.51, 109.53, 102.60, 94.44, 15.96 (2 × C). ESI-MS: m/z 384.4 (M + 1), 406.5 (M + 23). $C_{22}H_{17}N_5O_2$ (383.14).

*5.1.17. 6-((4-Cyanophenyl)amino)-N-(2-(dimethylamino)ethyl)-4-(mesityloxy)nicotinamide (**6b1**)*

White powder, yield: 69.4%. Mp: 208–210 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.75 (s, 1H, NH), 8.73 (s, 1H, pyridine-H), 8.20 (t, 1H, J = 5.1, NH), 7.86 (d, 2H, J = 8.8, PhH), 7.70 (d, 2H, J = 8.8, PhH), 7.06 (s, 2H, PhH), 5.94 (s, 1H, pyridine-H), 3.43 (q, 2H, J = 5.5, CH_2), 2.42 (t, 2H, J = 6.4, CH_2), 2.31 (s, 3H, CH_3), 2.13 (s, 6H, 2 × CH_3), 2.09 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.27, 162.63, 158.59, 152.34, 147.15, 145.66, 135.86, 133.52, 130.46, 130.31, 120.04, 118.42, 112.05, 102.43, 94.32, 58.12, 45.39 (2 × C), 37.79, 20.83, 16.01 (2 × C). ESI-MS: m/z 44.7 (M + 1). $C_{26}H_{29}N_5O_2$ (443.23).

*5.1.18. 6-((4-Cyanophenyl)amino)-4-(mesityloxy)-N-propylnicotinamide (**6b2**)*

White powder, yield: 69.7%. Mp: 224–225 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.71 (s, 1H, NH), 8.64 (s, 1H, pyridine-H), 8.12 (t, 1H, J = 5.2, NH), 7.84 (d, 2H, J = 8.8, PhH), 7.67 (d, 2H, J = 8.8, PhH), 7.06 (s, 2H, PhH), 5.92 (s, 1H, pyridine-H), 3.29 (q, 2H, J = 6.3, CH_2), 2.30 (s, 3H, CH_3), 2.08 (s, 6H, 2 × CH_3), 1.54 (q, 2H, J = 6.2, CH_2), 0.89 (t, 3H, J = 7.4, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.53, 162.57, 158.36, 151.73, 147.31, 145.73, 135.81, 133.54, 130.54, 130.28, 120.07, 118.33, 112.97, 102.32, 41.20, 22.92, 20.84, 16.12 (2 × C), 11.76. ESI-MS: m/z 415.6 (M + 1), 437.6 (M + 23). $C_{25}H_{26}N_4O_2$ (414.21).

*5.1.19. 6-((4-Cyanophenyl)amino)-4-(mesityloxy)-N-(2-methoxyethyl)nicotinamide (**6b3**)*

White powder, yield: 80.0%. Mp: 220–224 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.75 (s, 1H, NH), 8.72 (s, 1H, pyridine-H), 8.16 (t, 1H, J = 5.4, NH), 7.86 (d, 2H, J = 8.9, PhH), 7.68 (d, 2H, J = 8.8, PhH), 7.07 (s, 2H, PhH), 5.95 (s, 1H, pyridine-H), 3.53–3.48 (m, 4H, CH_2), 3.26 (s, 3H, CH_3), 2.31 (s, 3H, CH_3), 2.09 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.43, 162.62, 158.62, 152.31, 147.20, 145.65, 135.92, 133.53, 130.53, 130.31, 120.04, 118.43, 112.05, 102.45, 94.40, 71.02, 58.41, 39.19, 20.83, 16.05 (2 × C). ESI-MS: m/z 431.6 (M + 1), 453.5(M + 23). $C_{25}H_{26}N_4O_3$ (430.20).

*5.1.20. 6-((4-Cyanophenyl)amino)-N-(cyclopropylmethyl)-4-(mesityloxy)nicotinamide (**6b4**)*

White powder, yield: 70.6%. Mp: 228–230 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.73 (s, 1H, NH), 8.68 (s, 1H, pyridine-H), 8.17 (t, 1H, J = 5.7, NH), 7.85 (d, 2H, J = 8.8, PhH), 7.68 (d, 2H, J = 8.8, PhH), 7.06 (s, 2H, PhH), 5.94 (s, 1H, pyridine-H), 3.24 (t, 2H, J = 6.2, CH_2), 2.31 (s, 3H, CH_3), 2.09 (s, 6H, 2 × CH_3), 1.08–1.06 (m, 1H, CH), 0.43–0.39 (m, 2H, CH_2), 0.26–0.23 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.50, 162.59, 158.45, 151.99, 147.32, 145.71, 135.85, 133.54, 130.56, 130.30, 120.06, 118.37, 112.64, 102.36, 94.45, 43.50, 20.84, 16.13 (2 × C), 11.47, 3.44 (2 × C). ESI-MS: m/z 427.5 (M + 1), 449.5 (M + 23). $C_{26}H_{26}N_4O_2$ (426.21).

*5.1.21. 6-((4-Cyanophenyl)amino)-4-(mesityloxy)-N-(prop-2-yn-1-yl)nicotinamide (**6b5**)*

White powder, yield: 62.9%. Mp: 250–252 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.76 (s, 1H, NH), 8.72 (s, 1H, pyridine-H), 8.51 (t, 1H, J = 5.6, NH), 7.87 (d, 2H, J = 8.8, PhH), 7.69 (d, 2H, J = 8.8, PhH), 7.06 (s, 2H, PhH), 5.95 (s, 1H, pyridine-H), 4.13 (dd, 2H, J_1 = 4.6, J_2 = 2.4, CH_2), 3.09 (t, 1H, J = 2.4, CH), 2.31 (s, 3H, CH_3), 2.10 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.40, 162.69, 158.73, 152.33, 147.33, 145.62, 135.86, 133.53, 130.60, 130.26, 120.03, 118.48, 111.87, 102.52, 94.42, 82.13, 72.89, 29.11, 20.84, 16.17 (2 × C). ESI-MS: m/z 411.5 (M + 1), 433.6 (M + 23). $C_{25}H_{22}N_4O_2$ (410.17).

*5.1.22. 6-((4-Cyanophenyl)amino)-N-(2,2-dimethoxyethyl)-4-(mesityloxy)nicotinamide(**6b6**)*

White powder, yield: 62.3%. Mp: 222–224 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.74 (s, 1H, NH), 8.72 (s, 1H, pyridine-H), 8.09 (t, 1H, J = 5.7, NH), 7.86 (d, 2H, J = 8.8, PhH), 7.68 (d, 2H, J = 8.8, PhH), 7.07 (s, 2H, PhH), 5.95 (s, 1H, pyridine-H), 4.56 (t, 1H, J = 5.5, CH), 3.48 (t, 2H, J = 5.6, CH_2), 3.30 (s, 6H, 2 × CH_3), 2.31 (s, 3H, CH_3), 2.09 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.59, 162.67, 158.70, 152.38, 147.15, 145.62, 135.95, 133.52, 130.51, 130.33, 120.02, 118.48, 111.83, 102.52, 102.48, 94.38, 53.96 (2 × C), 41.31, 20.83, 16.02 (2 × C). ESI-MS: m/z 461.5 (M + 1), 483.5(M + 23). $C_{26}H_{28}N_4O_4$ (460.21).

*5.1.23. 6-((4-Cyanophenyl)amino)-N-cyclopropyl-4-(mesityloxy) nicotinamide (**6b7**)*

White powder, yield: 81.1%. Mp: 253–254 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.68 (s, 1H, NH), 8.52 (s, 1H, pyridine-H), 8.09 (d, 1H, J = 3.9, NH), 7.84 (d, 2H, J = 8.8, PhH), 7.67 (d, 2H, J = 8.8, PhH), 7.04 (s, 2H, PhH), 5.91 (s, 1H, pyridine-H), 2.90–2.85 (m, 1H, CH), 2.30 (s, 3H, CH_3), 2.06 (s, 6H, 2 × CH_3), 0.75–0.71 (m, 2H, CH_2), 0.59–0.57 (m, 2H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 165.13, 162.56, 158.23, 150.90, 147.40, 145.77, 135.72, 133.50, 130.51, 130.27, 120.03, 118.32, 113.77, 102.34, 94.65, 23.35, 20.81, 16.10, 6.69. ESI-MS: m/z 413.6(M + 1), 435.6(M + 23). $C_{25}H_{24}N_4O_2$ (412.19).

*5.1.24. 6-((4-Cyanophenyl)amino)-4-(mesityloxy)-N-((tetrahydrofuran-2-yl)methyl)nicotinamide(**6b8**)*

White powder, yield: 75.4%. Mp: 226–228 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.75 (s, 1H, NH), 8.71 (s, 1H, pyridine-H), 8.09 (t, 1H, J = 5.7, NH), 7.86 (d, J = 8.9, 2H, PhH), 7.68 (d, 2H, J = 8.8, PhH), 7.07 (s, 2H, PhH), 5.94 (s, 1H, pyridine-H), 4.00 (q, 1H, J = 6.0, CH), 3.70 (m, 1H, CH_2), 3.60 (m, 1H, CH_2), 3.44 (t, 2H, J = 5.6, CH_2), 2.31 (s, 3H, CH_3), 2.09 (s, 6H, 2 × CH_3), 1.93–1.86 (m, 1H, CH_2), 1.83–1.76 (m, 2H, CH_2), 1.63–1.58 (m, 1H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.59, 162.59, 158.62, 152.30, 147.11, 145.64, 135.97, 133.54, 130.49, 130.36, 120.04, 118.43, 112.04, 102.46, 94.38, 77.46, 67.77, 43.17, 28.69, 25.77, 20.83, 16.05. ESI-MS: m/z 457.6 (M + 1), 479.5(M + 23). $C_{27}H_{28}N_4O_3$ (456.22).

*5.1.25. N-allyl-6-((4-cyanophenyl)amino)-4-(mesityloxy) nicotinamide (**6b9**)*

White powder, yield: 74.0%. Mp: 207–210 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.73 (s, 1H, NH), 8.68 (s, 1H, pyridine-H), 8.27 (t, 1H, J = 5.8, NH), 7.86 (d, J = 8.9, 2H, PhH), 7.68 (d, 2H, J = 8.8, PhH), 7.06 (s, 2H, PhH), 5.93 (s, 1H, pyridine-H), 5,97–5.90 (m, 1H, C]CH), 5.20 (dd, 1H, J_1 = 18.8, J_2 = 1.7, C]CH_2) 5.09 (dd, 1H, J_1 = 12.0, J_2 = 1.5, C]CH_2), 4.00–3.97 (m, 2H, CH_2), 2.30 (s, 3H, CH_3), 2.09 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.47, 162.63, 158.49, 151.96, 147.27, 145.70, 136.08, 135.84, 133.53, 130.56, 130.29, 120.05, 118.39, 115.01, 112.62, 102.39, 94.42, 42.73, 20.83, 16.14 (2 × C). ESI-MS: m/z 413.6 (M + 1), 435.5 (M + 23). $C_{25}H_{24}N_4O_2$ (412.19).

*5.1.26. N-(2-cyanoethyl)-6-((4-cyanophenyl)amino)-4-(mesityloxy)nicotinamide (**6b10**)*

White powder, yield: 67.4%. Mp: 204–206 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.77 (s, 1H, NH), 8.74 (s, 1H, pyridine-H), 8.51 (t, 1H, J = 5.8, NH), 7.86 (d, J = 8.8, 2H, PhH), 7.68 (d, 2H, J = 8.8, PhH), 7.07 (s, 2H, PhH), 5.94 (s, 1H, pyridine-H), 3.60 (q, 2H, J = 6.3, CH_2), 2.82 (t, 2H, J = 6.4, CH_2), 2.31 (s, 3H, CH_3) 2.10 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 163.83, 162.76, 158.80, 152.52, 147.30, 145.60, 135.87, 133.54, 130.67, 130.23, 120.04, 119.70, 118.49, 111.55, 102.52, 94.29, 35.75, 20.84, 18.08, 16.16

(2 × C). ESI-MS: *m/z* 426.5 (M + 1), 448.5(M + 23). $C_{25}H_{23}N_5O_2$ (425.19).

5.1.27. 6-((4-Cyanophenyl)amino)-4-(mesityloxy)nicotinamide (**6b11**)

White powder, yield: 64.7%. Mp: >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 9.73 (s, 1H, NH), 8.70 (s, 1H, pyridine-H), 7.85 (d, 2H, J = 8.7, PhH), 7.68 (d, 2H, J = 8.7, PhH), 7.61 (s, 1H, NH), 7.55 (s, 1H, NH), 7.06 (s, 2H, PhH), 5.91 (s, 1H, pyridine-H), 2.30 (s, 3H, CH_3), 2.08 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ: 165.08, 162.81, 158.69, 152.50, 147.23, 145.67, 135.79, 133.55, 130.51, 130.30, 120.07, 118.40, 112.29, 102.38, 94.34, 20.84, 16.13 (2 × C). ESI-MS: *m/z* 373.3 (M + 1), 395.4(M + 23). $C_{22}H_{20}N_4O_2$ (372.16).

5.2. Biological activity

5.2.1. In vitro anti-HIV assay

The methodology of the anti-HIV assay has been previously described [19,20]. Stock solutions (10 × final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells 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 were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV- and mock-infected cell samples were included for each sample.

HIV-1 [24] (III_B, RES056) or HIV-2 [25] (ROD) stock (50 μL) at 100–300 $CCID_{50}$ (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 tested compound. 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, 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in a computer-controlled photometer (Infinite M1000, Tecan, Mechelen, Belgium), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of three 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 samples by 50%. The 50% effective concentration (EC_{50}) was defined as the compound concentration required for inhibiting virus-induced syncytium formation by 50%.

5.2.2. HIV-1 RT inhibition assay

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 [26]. Briefly, the reaction mixture consists of template/primer complex, 2′-deoxynucleotide-5′-triphosphates (dNTPs) and reverse transcriptase (RT) enzyme in the lysis buffer with or without inhibitors was incubated for 1 h at 37 °C. Then the reaction mixture was transferred to streptavidine-coated microtiter plate (MTP) and incubated for another 1 h at 37 °C. The biotin-labeled dNTPs that were incorporated in the template due to activity of RT were bound to streptavidine. The unbound dNTPs were washed using wash buffer and antidigoxigenin-peroxidase (DIG-POD) was added to the MTP. The DIG-labeled dNTPs incorporated in the template was 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 the cleavage of the substrate catalyzed y a peroxide enzyme. The absorbance of the sample was determined at OD 405 nM using microtiter plate ELISA reader. The percentage inhibitory activity of RT inhibitors was calculated by comparison to a sample that did not contain an inhibitor. The percentage inhibition was calculated by formula as given below: % inhibition = 100 − (OD 405 nm without inhibitor − OD405 nm with inhibitor)/(OD405 nm without inhibitor − OD405 nm background) × 100. IC_{50} values corresponded to the concentration of the tested compounds required to inhibit biotin-dUTP incorporation into the HIV-1 RT by 50%.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.054.

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