CHEMMEDCHEM

DOI: 10.1002/cmdc.201000555

Synthesis and Biological Evaluation of 6-Substituted 5-Alkyl-2-(phenylaminocarbonylmethylthio)pyrimidin-4(3*H*)-ones as Potent HIV-1 NNRTIs

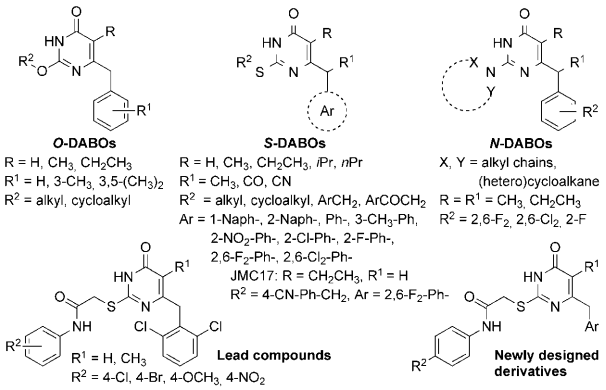
Mingyan Yu,[a] Zhenyu Li,[a] Shuai Liu,[a] Erkang Fan,[b] Christophe Pannecouque,[c] Erik De Clercq,[c] and Xinyong Liu*[a]

A series of new 5-alkyl-2-phenylaminocarbonylmethylthiopyrimidin-4(3*H*)-ones bearing variously substituted arylmethyl moieties at the C6 position of the pyrimidine ring were synthesized and evaluated for anti-HIV activity in MT-4 cells. Most of these new congeners exhibited moderate to good activities against the wild-type virus, with EC_{50} values in the range of 1.40–0.19 μM. Among them, 2-[(4-cyanophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one **4b6** is one of the compounds endowed with the highest broad-spectrum HIV-1 inhibitory activity, with EC_{50} values of 0.19 ± 0.005 μM against the wild-type virus, 1.05 ± 0.24 μM (twofold resistance) against the E138K strain, and 2.38 ± 0.13 μM (4.5-fold resistance) against the Y181C strain. Furthermore, reverse transcriptase (RT) inhibition assays against wild-type HIV-1 RT were performed with selected derivatives, confirming that the main target of these compounds is HIV-1 RT and that these new *S*-DABO analogues act as non-nucleoside RT inhibitors (NNRTIs). Structure–activity relationship and molecular modeling analyses of these new congeners are also discussed.

Introduction

Human immunodeficiency virus (HIV) infection affects nearly 60 million individuals worldwide. Since the first cases of individuals dying from a rare opportunistic infection was reported, 25 million people have died from this epidemic.[1] Although the introduction of highly active antiretroviral therapy (HAART) has dramatically decreased the morbidity and mortality resulting from the infection with HIV, AIDS has remained one of the world's most serious health problems.[2] In the research of anti-HIV agents, HIV-1 non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to an allosteric site on reverse transcriptase (RT) and represent an important therapeutic class of inhibitors used in the treatment of HIV-1 infection.[3–5] Among the NNRTIs, dihydroalkyloxybenzyloxopyrimidines (DABOs) were reported as potent HIV NNRTIs, with robust anti-HIV-1 activity against both the wild-type (wt) and a panel of clinically relevant HIV-1 mutants.[6] Three generations of DABO analogues have been found to date: dihydroalkyloxybenzyloxopyrimidines (*O*-DABOs), dihydroalkylthiobenzyloxopyrimidines (*S*-DABOs), and dihydroalkylaminodifluorobenzyloxopyrimidines (*N*-DABOs), [7–11] from which many promising DABOs have been developed.

Our recent work on *S*-DABOs disclosed that assembly of a phenylaminocarbonylmethylthio moiety at the C2 position of the pyrimidine ring has resulted in new lead compounds with potent antiviral activities against HIV-1.[12] Continuing our research to obtain new SAR information and to explore the effect of the insertion of a C2-phenylaminocarbonylmethylthio moiety in *S*-DABOs, we designed a novel series of *S*-DABOs with structural characterization of a 2-phenylaminocarbonylmethylthio chain at C2, a 2-chloro-6-fluoro-, 1-naphthylmethyl, or 2,6-difluorobenzyl group at C6, and a methyl or ethyl substituent at C5. Herein we describe the synthesis, anti-HIV-1 activity, and preliminary SAR studies of these new congeners. The different degrees of potency displayed by the new molecules against wild-type and mutant HIV-1 virus were also studied by molecular modeling approaches.



[a] *Dr. M. Yu, Z. Li, S. Liu, Prof. Dr. X. Liu*
Institute of Medicinal Chemistry, School of Pharmaceutical Sciences
Shandong University, No. 44 Wenhuaxi Road, Jinan 250012 (P.R. China)
Fax: (+86) 531 88382731
E-mail: xinyongl@sdu.edu.cn

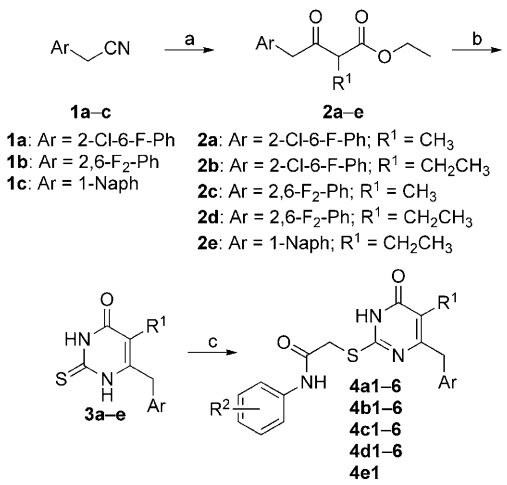
[b] *E. Fan*
Biomolecular Structure Center
University of Washington, 98195, Seattle, WA (USA)

[c] *Prof. Dr. C. Pannecouque, Prof. Dr. E. De Clercq*
Rega Institute for Medical Research
Katholieke Universiteit Leuven, 3000 Leuven (Belgium)

Results and Discussion

Chemistry

The target compounds **4a1**–**4a6**, **4b1**–**4b6**, **4c1**–**4c6**, **4d1**–**4d6**, and **4e1** were prepared as depicted in Scheme 1. The β-keto esters **2a**–**e** were prepared by a simple and high-yielding



Scheme 1. *Reagents and conditions*: a) 1. $R^1CHBrCOOEt$, Zn, THF, reflux, 2. K_2CO_3 (50%), 3. HCl (13%); b) thiourea, NaOEt, reflux, 6–12 h; c) appropriate *N*-phenylacetamide halides, K_2CO_3, DMF, room temperature, 12 h.

method reported by Hannick and Kishi through the reaction of arylacetonitriles **1a**–**c** with activated zinc dust and 2-bromoalkanoates.[13,14] Subsequent condensation of β-keto esters **2a**–**e** with thiourea in the presence of sodium ethoxide in ethanol at reflux gave the key intermediates 5-alkyl-6-substituted thiouracil **3a**–**e**.[15] Next, selective *S*-alkylation of **3a**–**e** with the appropriate *N*-phenylacetamide halides (1:1.1) in the presence of potassium carbonate in anhydrous *N*,*N*-dimethylformamide (DMF) afforded the desired target compounds **4a1**–**4a6**, **4b1**–**4b6**, **4c1**–**4c6**, **4d1**–**4d6**, and **4e1**. Both analytical and spectral data for all compounds are in full agreement with the proposed structures.

Anti-HIV activity in MT-4 cells

According to the MTT method,[16,17] the newly designed and synthesized *S*-DABO analogues (compounds **4a**–**e**) were tested in MT-4 cells to evaluate their biological activity against wt HIV-1 strain III_B and HIV-2 strain ROD. The results, expressed as EC_{50}, CC_{50}, and SI (selectivity index) values, are listed in Table 1. Nevirapine (NVP), zidovudine (azidothymidine, AZT), dideoxycytidine (DDC), and dideoxyinosine (DDI) were used as reference drugs. Selected compounds were also tested against HIV-1 mutant RT strains E138K, F227L+V106A, K103N, L100I, RES056 (K103N+Y181C), Y181C, and Y188L. The results are listed in Table 2 together with those of NVP, AZT, efavirenz (EFV), delavirdine (DLV), and etravirine (TMC125) as reference standards.

The results of the anti-HIV assay demonstrated that the majority of these series of compounds exhibited moderate to good activities against HIV-1 strain III_B with EC_{50} values in the range of 1.40–0.19 μM, whereas compounds **4b3** and **4b4** were inactive. The most active *S*-DABO derivative was compound **4b6** with an EC_{50} value of 0.19 ± 0.005 μM and a SI value of >169, which are much better than those of NVP, DDC, and DDI. It is worth noting that the SI values of these *S*-DABO derivatives are very variable, ranging from 10 to 785. Compound **4a2** was identified as the most selective compound in this series (SI = 785), which is also much more selective than NVP, DDC, and DDI.

The SAR analysis showed that the combined action of the substituents at the C5 position and C6 position of the pyrimidine ring influenced the anti-HIV-1 strain III_B activity of the new *S*-DABO analogues. When C5 was substituted by a methyl group, the 2-chloro-6-fluorobenzyl analogues (**4a1**–**4a6**) were more potent than the corresponding 2,6-difluorobenzyl analogues (**4c1**–**4c6**) in inhibiting wt HIV-1, with the exception of **4a1**. However, when C5 was substituted by an ethyl moiety, the 2,6-difluorobenzyl analogues **4d1**–**4d6** were more potent than the corresponding 2-chloro-6-fluoro analogues **4b1**–**4b6** with the exception of **4d5** and **4d6**. Moreover, it was further confirmed that the substituent at the C5 position of the pyrimidine ring also influenced the anti-HIV-1 activity of these new congeners. When the C5 substituents were changed from methyl to ethyl, a marked increase in anti-HIV-1 activity was observed for the 2,6-difluorobenzyl analogues (**4c1**–**4c6** versus **4d1**–**4d6**). The above observations contrasted sharply with the 2-chloro-6-fluorobenzyl derivatives (**4a1**–**4a6** versus **4b1**–**4b6**), in which the steric bulk of the C5 substituent is detrimental to HIV-1 inhibitory activity. In addition, when the C6 group was substituted by 1-naphthylmethyl (**4e1**), the compound lost its anti-HIV-1 activity completely.

In fact, the variable activities of these novel series compounds are attributed to the changed substituent at C2, in which a phenylmethylthio group of the *S*-DABOs prototypes was replaced by the phenylaminocarbonylmethylthio group. The presence of a C2 phenylaminocarbonylmethylthio moiety changes the original orientation of the *S*-DABOs in the non-nucleoside binding site (NNBS) of HIV-1 RT. This issue is addressed in the section concerning molecular modeling analysis.

In addition, the 15 selected compounds were also tested against a panel of HIV-1 mutant RT strains E138K, F227L+V106A, K103N, L100I, RES056 (K103N+Y181C), Y181C, and Y188L (Table 2). To define the resistance profile of these compounds, both the absolute activity against the HIV-1 mutants (EC_{50} values) and the relative activity (fold resistance) need to be considered. In this assay, the selected compounds retained, in part, their activities against the E138K and Y181C mutant strains, with EC_{50} values at the low micromolar level, whereas they were inactive against the HIV-1 F227L+V106A, K103N, L100I, RES056 (K103N+Y181C), and Y188L mutant strains. Their fold resistance values ranged from 2 to 23.8 (E138K), and from 4.5 to 97 (Y181C). Among the tested compounds, **4d6** was identified as the most active compound, having moderate activity against mutant strains E138K (EC_{50} 1.05 ± 0.24 μM with

Table 1. Anti-HIV activities of compounds **4a–e** against wt HIV-1 III_B and HIV-2 ROD strains in MT-4 cells.

Compd	Ar	R^1	R^2	EC_{50} [μM][a] HIV-1 III_B	HIV-2 ROD	CC_{50} [μM][b]	SI (III_B)[c]
4a1	2-Cl-6-F-Ph	$-CH_3$	-F	0.77 ± 0.49	> 152.80	152.80	≥ 199
4a2	2-Cl-6-F-Ph	$-CH_3$	-Cl	0.27 ± 0.03	> 210.80	210.80 ± 78.13	785
4a3	2-Cl-6-F-Ph	$-CH_3$	-Br	0.30 ± 0.05	> 143.26	143.26 ± 38.48	479
4a4	2-Cl-6-F-Ph	$-CH_3$	$-OCH_3$	0.44 ± 0.32	> 10.25	10.24 ± 4.43	23
4a5	2-Cl-6-F-Ph	$-CH_3$	$-NO_2$	1.14 ± 0.01	> 20.33	20.33 ± 4.84	18
4a6	2-Cl-6-F-Ph	$-CH_3$	-CN	0.45 ± 0.24	> 73.15	73.15 ± 62.29	162
4b1	2-Cl-6-F-Ph	$-CH_2CH_3$	-F	1.26 ± 0.17	> 277.84	277.84	> 220
4b2	2-Cl-6-F-Ph	$-CH_2CH_3$	-Cl	0.50 ± 0.32	> 5.10	5.09 ± 0.74	10
4b3	2-Cl-6-F-Ph	$-CH_2CH_3$	-Br	≥ 0.65	> 2.35	2.35 ± 0.97	≤ 4
4b4	2-Cl-6-F-Ph	$-CH_2CH_3$	$-OCH_3$	> 3.23	> 3.23	3.22 ± 0.85	< 1
4b5	2-Cl-6-F-Ph	$-CH_2CH_3$	$-NO_2$	0.39 ± 0.15	> 23.02	23.01 ± 1.81	60
4b6	2-Cl-6-F-Ph	$-CH_2CH_3$	-CN	0.19 ± 0.005	> 31.84	31.84 ± 1.11	169
4c1	2,6-F_2-Ph	$-CH_3$	-F	0.61 ± 0.08	> 144.72	144.72 ± 34.48	237
4c2	2,6-F_2-Ph	$-CH_3$	-Cl	0.41 ± 0.14	> 82.94	82.94 ± 16.02	200
4c3	2,6-F_2-Ph	$-CH_3$	-Br	0.60 ± 0.18	> 96.24	96.24 ± 9.20	160
4c4	2,6-F_2-Ph	$-CH_3$	$-OCH_3$	0.79 ± 0.29	> 163.40	163.40 ± 50.89	208
4c5	2,6-F_2-Ph	$-CH_3$	$-NO_2$	1.40 ± 0.40	> 167.33	167.33 ± 19.80	120
4c6	2,6-F_2-Ph	$-CH_3$	-CN	0.56 ± 0.11	> 129.80	129.80 ± 13.50	231
4d1	2,6-F_2-Ph	$-CH_2CH_3$	-F	0.31 ± 0.04	> 178.98	178.97 ± 36.83	575
4d2	2,6-F_2-Ph	$-CH_2CH_3$	-Cl	0.21 ± 0.02	> 53.97	53.96 ± 31.45	251
4d3	2,6-F_2-Ph	$-CH_2CH_3$	-Br	0.29 ± 0.07	> 4.85	4.85 ± 0.59	17
4d4	2,6-F_2-Ph	$-CH_2CH_3$	$-OCH_3$	0.27 ± 0.08	> 3.59	3.59 ± 0.28	13
4d5	2,6-F_2-Ph	$-CH_2CH_3$	$-NO_2$	1.03 ± 0.27	> 152.03	152.03 ± 34.46	148
4d6	2,6-F_2-Ph	$-CH_2CH_3$	-CN	0.48 ± 0.39	> 164.55	164.54 ± 82.34	344
4e1	1-Naph	$-CH_2CH_3$	-F	> 44.76	> 44.76	44.77 ± 10.61	< 1
NVP				0.19 ± 0.04	> 15	> 15	> 80
AZT				0.0076 ± 0.0014	0.0033 ± 0.0006	> 88.26	> 11 587
DDC				0.75 ± 0.55	0.88 ± 0.53	> 94	> 127
DDI				8.86 ± 2.89	16 ± 5.16	> 211	> 13

[a] Compound concentration required to effect 50% protection of MT-4 cells against HIV-induced cytotoxicity, as determined by the MTT method. [b] Compound concentration required to decrease the viability of mock-infected cells by 50%, as determined by the MTT method. [c] Selectivity index: CC_{50}/EC_{50} ratio (wt HIV-1 III_B).

Table 2. Anti-HIV-1 activity of selected compounds against HIV-1 mutant strains.

Compd	EC_{50} [μM][a] (fold resistance)[b] E138K	Y181C
4a1	3.87 ± 0.86 (6.1)	> 152.80 (> 240.4)
4a2	2.68 ± 0.25 (7.7)	> 210.80 (> 610.2)
4a3	2.51 ± 0.27 (7.5)	32.41 ± 9.11 (97)
4a6	2.07 ± 0.97 (4.9)	15.83 ± 3.10 (37.3)
4b1	4.19 ± 1.90 (2.6)	9.85 ± 2.54 (6.2)
4b5	2.62 ± 0.24 (6.4)	2.54 ± 0.62 (6.2)
4b6	2.03 ± 0.3 (7.2)	2.13 ± 0.58 (7.6)
4c2	10.26 ± 8.84 (23.8)	> 82.94 (> 192.0)
4c3	12.13 ± 12.07 (22)	> 96.24 (> 175.0)
4c4	3.78 ± 0.62 (5.2)	152.97 (> 212.9)
4c6	3.76 ± 0.75 (7.1)	> 129.80 (> 246.5)
4d1	2.13 ± 0.02 (5.3)	> 178.98 (> 443.9)
4d2	1.64 ± 0.59 (8.2)	> 53.97 (> 311.9)
4d3	≥ 1.03 (≥ 3.7)	> 4.85 (> 17.2)
4d6	1.05 ± 0.24 (2)	2.38 ± 0.13 (4.5)
NVP	0.18 ± 0.057 (1.2)	NA
AZT	0.02 ± 0.009 (1.4)	0.0079 ± 0.0012(0.5)
EFV	0.0055 ± 0.00078 (1.4)	0.0055 ± 0.000479 (1.4)
DLV	0.06 ± 0.023 (1.9)	1.47 ± 0.33 (45.4)
TMC125	0.008 ± 0.002 (2.6)	0.009 ± 0.0048 (3.2)

[a] Compound concentration required to effect 50% protection of MT-4 cells against cytopathicity induced by HIV-1 mutant strains, as determined by the MTT method. [b] Ratio of EC_{50} value against drug-resistant strain and EC_{50} of wt HIV-1 III_B ($EC_{50}^{mut}/EC_{50}^{wt}$).

a twofold resistance ratio with respect to wt) and Y181C (EC_{50} 2.38 ± 0.13 μM with a 4.5-fold resistance ratio). Compared with 2,6-difluorobenzyl analogues, the 2-chloro-6-fluorobenzyl analogues showed a lower fold resistance of antiviral activity from wt to E138K and Y181C HIV-1 strains (with the exception of **4b6** versus **4d6**). Furthermore, the newly synthesized compounds **4a–e** were also evaluated for their capacity to inhibit HIV-2 (strain ROD) replication in MT-4 cells, but none were found to be effective (Table 1).

Inhibition of HIV-1 RT_{wt}

To directly prove that the newly synthesized compounds target HIV-1 RT, we chose two compounds (**4b6** and **4d2**) to evaluate HIV-1 RT inhibitory activity, 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, and nevirapine as a reference compound (Table 3). The results show that these new compounds do inhibit the activity of HIV-1 RT_{wt} with IC_{50} values of 3.89 and 16.8 μM for **4b6** and **4d2**, respectively. In addition, none of the newly synthesized compounds was active against HIV-2 (ROD) in the cell assay. Based on the chemical structure and the general characteristic that NNRTIs inhibit HIV-1 but not HIV-2 replication, it could be con-

Table 3. Selected compounds assayed against wt HIV-1 RT.

Compd	IC_{50} [μM][a]
4b6	3.89
4d2	16.8
NVP	0.30

[a] Compound concentration required to inhibit HIV-1 RT_{wt} activity by 50%.

cluded that the newly synthesized *S*-DABO compounds targeted the HIV-1 RT, therefore acting as NNRTIs.

Molecular modeling analysis

To investigate the binding modes of the newly synthesized compounds, molecular modeling study was performed by means of AutoDock Vina for docking. Default parameters were used as described in the AutoDock Vina manual unless otherwise specified.

Derivative **4b6** was identified as the most potent compound against wt and mutant HIV-1 strains. Thus, **4b6** was taken as a representative compound to study the 2-phenylaminocarbonylmethyl-*S*-DABO binding models by docking into the NNBS of either RT_{wt} or RT_{Y181C}. To check the structural role of substitution at the C2 side chain of *S*-DABO, the same docking procedure was carried out on a structurally related *S*-DABO (JMC17)[6] and also on TNK-651, a potent 1-[(2-hydroxyethoxy)-methyl]-6-(phenylthio)thymine (HEPT) analogue. The coordinates of TNK-651 co-crystallized in either RT_{wt} (PDB code: 1RT2)[18] or RT_{Y181C} (PDB code: 1JLA)[19] were used to define the $NNBS_{wt}$ and $NNBS_{Y181C}$. The docking modes are shown in Figure 1.

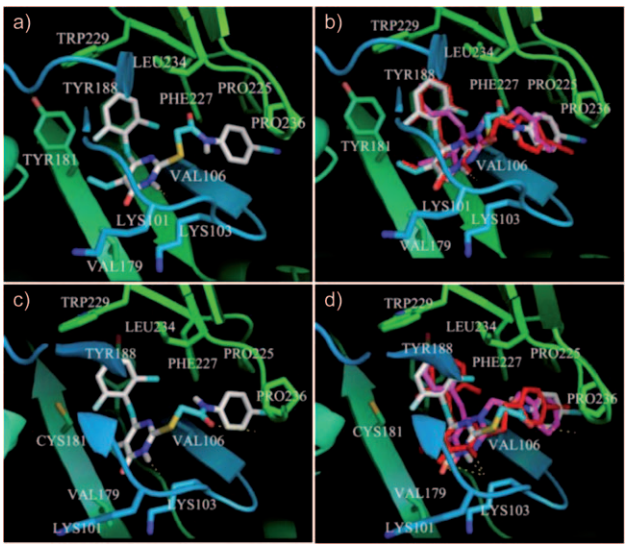


Figure 1. a) Model of **4b6** docked into RT_{wt} (PDB code: 1RT2). b) Superimposition of the docked conformations of **4b6** (white), JMC17 (red), and TNK-651 (purple) in RT_{wt} (PDB code: 1RT2). c) Model of **4b6** docked into RT_{Y181C} (PDB code: 1JLA). d) Superimposition of the docked conformations of **4b6** (white), JMC17 (red), and TNK-651 (purple) in RT_{Y181C} (PDB code: 1JLA)

The analyses of the **4b6** docking results revealed that the overall binding mode is not influenced by the Y181C mutation. The two binding modes (Figure 1a, Figure 1c) show the following: 1) The 2-phenylaminocarbonylmethylthio substituent is well accommodated in the large pocket mainly defined by Val106, Pro225, Pro236, and Phe227. A hydrogen bond is formed between the C=O group of the C2 side chain and surrounding amino acids only in RT_{Y181C}. Lengthening the C2 side chain leads the C2 side chain to go beyond the opening defined by Pro225 and Pro236. Thus, the end of C2 side chain is exposed to the solvent, causing the lack of its hydrophobic interactions with the NNIBP. 2) The pyrimidine NH moiety at position 3 is engaged in a hydrogen bond with the C=O moiety of Lys101. 3) The alkyl group at the C5 position is positioned in the hydrophobic cavity formed by the Val179 side chains. 4) The benzyl substituent at C6 of the pyrimidine ring is accommodated in a hydrophobic pocket mainly defined by the aromatic side chains of Tyr181 (Cys181), Tyr188, Phe227, and Trp229 as well as by Leu234. In particular, the phenyl ring interacts favorably with the Tyr188 side chain, giving rise to a positive π-stacking interaction.

Inspection of the binding modes of **4b6** reveals a fair superimposition with those of JMC17 and TNK-651 both in RT_{wt} and RT_{Y181C}, showing an overall common binding conformation (Figure 1b, Figure 1c). Notably, all compounds share the same binding modes in RT_{wt} (Figure 1b). Although the three compounds are still superimposable in RT_{Y181C}, the binding conformations of the compounds are slightly less overlapped. In particular, the benzyl substituent at C6 of **4b6** does not overlap the C6 position of compounds JMC17 and TNK-651 (Figure 1d). These results appear to be due to the introduction, in **4b6**, of a new anchor point (C=O at C2 side chain) by forming a new hydrogen bond with NNBP. The new hydrogen bond pattern changes the original orientation of the *S*-ADBOs in the RT_{Y181C} (Figure 1d). Meanwhile, the new hydrogen bond with NNBP at the C2 side chain could compensate for the incoming lack of positive hydrophobic ligand–NNBP interactions due to the Tyr181 to Cys181 mutation. As a result, **4b6** could be expected to be less susceptible than JMC17 to decreasing activity when tested against the Y181C mutated strain of HIV-1. This resistance profile of **4b6** (4.5-fold resistance) is slightly better than that of JMC17 (17.3-fold resistance).[6]

Thus, the 2-phenylaminocarbonylmethyl-*S*-DABOs can be considered as promising novel DABO candidates for further development, and for further studies aimed at evaluating their effectiveness as anti-AIDS agents that are active against both wild-type and mutated strains of HIV-1.

Conclusions

We designed and synthesized a series of novel 5-alkyl-6-arylmethyl-2-phenylaminocarbonylmethylthio substituted DABOs, which were evaluated for their biological activity against wild-type HIV-1 (III_B strain) and HIV-2 (ROD strain) in MT-4 cells. Selected compounds were also tested against HIV-1 mutant virus strains E138K, F227L+V106A, K103N, L100I, RES056 (K103N+Y181C), Y181C, and Y188L. Some of the new compounds dis-

played anti-wt-HIV-1 activity at low micromolar concentrations along with low cytotoxicity. Among them, the most potent wt HIV-1 inhibitor is **4b6** ($EC_{50} = 0.19 \pm 0.005$ μM), which is more effective than the reference drugs NVP, DDC, and DDI. In addition, **4b6** retains potency against the E138K and Y181C mutants ($EC_{50}^{E138K} = 1.05 \pm 0.24$ μM, twofold resistance; $EC_{50}^{Y181C} = 2.38 \pm 0.13$ μM, 4.5-fold resistance). Furthermore, enzyme inhibitory assays were performed with selected derivatives against HIV-1 RT_{wt}. The results showed that the main target of these compounds is HIV-1 RT and these compounds act as NNRTIs. The preliminary SAR among the newly synthesized congeners and docking studies provided useful indications for guiding the further rational design of new *S*-DABO analogues as more active and selective HIV-1 inhibitors.

Experimental Section

Chemistry

All melting points were determined on a micromelting point apparatus and are uncorrected. IR spectra were recorded with a Nexus 470 FTIR spectrometer. NMR spectra were obtained on a Bruker Avance 600 NMR spectrometer in the indicated solvents. Chemical shift values (δ) are expressed in ppm, and $(CH_3)_4Si$ was used as internal reference. MS data were collected on an LC Autosampler device: Standard G1313A instrument. TLC was performed on silica gel GF_{254} (Merck), and spots were visualized by I_2 vapors or by irradiation with UV light (λ 254 nm). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure.

General procedure for the preparation of β-keto esters 2a–e: The activated Zn dust was prepared by washing Zn dust sequentially with 3 N $HCl_{(aq)}$, distilled H_2O, EtOH, Et_2O, and drying under vacuum. Activated Zn dust (32.5 g, 500 mmol) was suspended in dry THF (200 mL) and held at reflux under a N_2 atmosphere. A few drops of 2-bromoalkanoates were added to initiate the reaction. After the appearance of a green color, arylacetonitriles (100 mmol) were added in one portion followed by the dropwise addition of 2-bromoalkanoates (260 mmol) over 1 h. The reaction mixture was held at reflux for an additional 2 h, diluted with THF (600 mL), and quenched with $K_2CO_{3(aq)}$ (50%, 125 mL). Rapid stirring for 60 min gave two distinct layers. The THF layer was decanted, the residue washed with THF (150 mL), and the combined THF fractions were treated with $HCl_{(aq)}$ (10%, 150 mL) at room temperature for 45 min. The mixture was concentrated under reduced pressure, diluted with CH_2Cl_2 (300 mL), and washed with saturated $NaHCO_3$ (3× 350 mL) and brine (3×350 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated to give the crude products **2a–e**, which were directly used in the following step without further purification.

General procedure for the preparation of 5-alkyl-6-substituted thiouracil 3a–e: Na^0 (8.2 g, 356 mmol) was dissolved in absolute EtOH (50 mL), and thiourea (19 g, 249 mmol) and β-keto esters **2a–e** (178 mmol) were added to the clear solution at room temperature. The reaction mixture was held at reflux for 6–12 h (checked by TLC) under a N_2 atmosphere. The reaction mixture was cooled to room temperature. Then, solvent was evaporated and the residues were dissolved in H_2O and were precipitated by addition of concentrated $HCl_{(aq)}$ and subsequent acidification to pH 4 with glacial AcOH. The resulting precipitate was filtered under reduced pressure. The solid was washed sequentially with H_2O, EtOH, and Et_2O, then dried to give **3a–e**, which is directly used in the next step without further purification.

General procedure for the preparation of target compounds 4a–e: Compounds **3a–e** (5 mmol) and appropriate *N*-phenylacetamide halides (5.5 mmol) were suspended in anhydrous DMF (25 mL) in the presence of anhydrous K_2CO_3 (0.759 g, 5.5 mmol) at room temperature. The mixtures were irradiated at room temperature for 12 h. The reaction mixture was poured into cold H_2O (200 mL), the resulting precipitate was collected by filtration under reduced pressure and washed sequentially with H_2O, EtOH, and Et_2O, and then dried to give the corresponding crude product, which was purified by crystallization to give the pure target compounds **4a–e**.

2-[(4-Fluorophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4a1): Recrystallized from EtOH/DMF as a white crystal, Yield: 23.6%; mp: 237–239 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta = 12.74$ (s, 1H, NH), 9.95 (s, 1H, NH), 7.52–7.00 (m, 7H), 3.97 (s, 2H, *S*-CH_2), 3.74 (s, 2H, CH_2), 2.03 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu} = 3251(\upsilon_{NH})$, 3050 ($\upsilon_{NH}$), 1671($\upsilon_{C=O}$), 1654($\upsilon_{C=O}$), 1228 ($\upsilon_{C-N}$), 1213 cm^{-1} ($\upsilon_{C-N}$); ESI-MS: *m/z* 436.1 $[M+1]^+$, 458.1 $[M+Na]^+$; $C_{20}H_{16}ClF_2N_3O_2S$ (435.06).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4a2): Recrystallized from EtOH/DMF as a white crystal, Yield: 22.9%; mp: 238–240 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta = 12.74$ (s, 1H, NH), 10.04 (s, 1H, NH), 7.50 (d, $J = 8.4$ Hz, 2H), 7.37 (d, $J = 8.4$ Hz, 2H), 7.11–7.00 (m, 3H), 3.96 (s, 2H, *S*-CH_2), 3.75 (s, 2H, CH_2), 2.02 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu} = 3245(\upsilon_{NH})$, 3048($\upsilon_{NH}$), 1670($\upsilon_{C=O}$), 1656($\upsilon_{C=O}$), 1262 ($\upsilon_{C-N}$), 1245 cm^{-1} ($\upsilon_{C-N}$); ESI-MS: *m/z* 452.1 $[M+1]^+$, 474.1 $[M+Na]^+$; $C_{20}H_{16}Cl_2FN_3O_2S$ (451.03).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4a3): Recrystallized from EtOH/DMF as a white crystal, Yield: 28.6%; mp: 234–236 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta = 12.75$ (s, 1H, NH), 10.04 (s, 1H, NH), 7.50–6.99(m, 7H), 3.95 (s, 2H, *S*-CH_2), 3.75 (s, 2H, CH_2), 2.02 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu} = 3246(\upsilon_{NH})$, 3047($\upsilon_{NH}$), 1670($\upsilon_{C=O}$), 1657($\upsilon_{C=O}$), 1261 ($\upsilon_{C-N}$), 1245 cm^{-1} ($\upsilon_{C-N}$); ESI-MS: *m/z* 497.9 $[M+1]^+$, 520.0 $[M+Na]^+$; $C_{20}H_{16}BrClFN_3O_2S$ (496.98).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4a4): Recrystallized from EtOH/DMF as a white crystal, Yield: 26.3%;mp: 240–242 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta = 12.73$(s, 1H, NH), 10.30 (s, 1H, NH), 7.41–6.88(m, 7H), 3.98 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 3.71(s, OCH_3, 3H), 2.02 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu} = 3255(\upsilon_{NH})$, 3045($\upsilon_{NH}$), 1656($\upsilon_{C=O}$), 1247 cm^{-1} ($\upsilon_{C-N}$); ESI-MS: *m/z* 448.1 $[M+1]^+$, 470.1 $[M+Na]^+$; $C_{21}H_{19}ClFN_3O_3S$ (447. 08).

2-[(4-nitrophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4a5): Recrystallized from EtOH/DMF as a white crystal, Yield: 25.2%; mp: 235–237 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta = 12.71$ (s, 1H, NH), 10.54 (s, 1H, NH), 8.23 (d, $J = 9.0$ Hz, 2H), 7.74 (d, $J = 9.0$ Hz, 2H), 7.03–6.94 (m, 3H), 3.90 (s, 2H, *S*-CH_2), 3.83 (s, 2H, CH_2), 2.02 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu} = 3324(\upsilon_{NH})$, 3080($\upsilon_{NH}$), 1644($\upsilon_{C=O}$), 1249 cm^{-1} ($\upsilon_{C-N}$); ESI-MS: *m/z* 463.4 $[M+1]^+$, 485.5 $[M+Na]^+$; $C_{20}H_{16}ClFN_4O_4S$ (462.06).

2-[(4-cyanophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4a6): Recrystallized from EtOH/DMF as a white crystal, Yield: 24.8%; mp: 243–245 °C (dec); ^{1}H NMR ([D$_6$]DMSO): $\delta = 12.77$ (s, 1H, NH), 10.37(s, 1H, NH), 7.79 (d, $J = 9.0$ Hz, 2H), 7.68 (d, $J = 9.0$ Hz, 2H), 7.04–6.95 (m, 3H), 3.94 (s,

2H, *S*-CH_2), 3.80 (s, 2H, CH_2), 2.02 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3253(υ_{NH}), 3049(υ_{NH}), 2228 ($\upsilon_{C\equiv N}$), 1639($\upsilon_{C=O}$), 1245 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 443.6 $[M+1]^+$, 465.3 $[M+Na]^+$; $C_{21}H_{16}ClFN_4O_2S$ (442.07).

2-[(4-fluorophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4b1): Recrystallized from EtOH/DMF as a white crystal, Yield: 25.1%; mp: 212–214 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.71 (s, 1H, NH), 9.94 (s, 1H, NH), 7.50 (d, *J*= 9.0 Hz, 2H), 7.16 (d, *J*= 9.0 Hz, 2H), 7.13–7.01 (m, 3H), 3.99 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 2.51 (q, *J*= 7.2 Hz, CH_2CH_3, 2H), 1.04 ppm (t, *J*= 7.2 Hz, CH_2CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3252 (υ_{NH}), 3043(υ_{NH}), 1658($\upsilon_{C=O}$), 1245 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 450.1 $[M+1]^+$, 472.1 $[M+Na]^+$; $C_{21}H_{18}ClF_2N_3O_2S$ (449.08).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4b2): Recrystallized from EtOH/DMF as a white crystal, Yield: 25.6%; mp: 215–217 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.74 (s, 1H, NH), 10.61(s, 1H, NH), 7.53–7.00 (m, 7H), 4.04 (s, 2H, *S*-CH_2), 3.73 (s, 2H, CH_2), 2.52 (q, CH_2CH_3, 2H), 1.04 ppm (t, CH_2CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3289 (υ_{NH}), 3056 (υ_{NH}), 1648($\upsilon_{C=O}$), 1241 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 466.1 $[M+1]^+$, 488.1 $[M+Na]^+$; $C_{21}H_{18}Cl_2FN_3O_2S$ (465.05).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4b3): Recrystallized from EtOH/DMF as a white crystal, Yield: 24.1%; mp: 207–209 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.73 (s, 1H, NH), 10.20 (s, 1H, NH), 7.57–7.02 (m, 7H), 4.04 (s, 2H, *S*-CH_2), 3.79 (s, 2H, CH_2), 2.52(q, *J*=7.8 Hz, CH_2CH_3, 2H), 1.04 ppm (t, *J*= 7.8 Hz, CH_2CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3253 (υ_{NH}), 303n8(υ_{NH}), 1655 ($\upsilon_{C=O}$), 1245 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 512.5 $[M+1]^+$, 534.3 $[M+Na]^+$; $C_{21}H_{18}BrClFN_3O_2S$ (511.00).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4b4): Recrystallized from EtOH/DMF as a white crystal, Yield: 28.4%; mp: 241–243 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.72 (s, 1H, NH), 9.77(s, 1H, NH), 7.41–6.88 (m, 7H), 4.01 (s, 2H, *S*-CH_2), 3.72 (s, 2H, CH_2), 3.69 (s, 3H, OCH_3), 2.51(q, *J*=7.2 Hz, CH_2CH_3, 2H), 1.04 ppm (t, *J*= 7.2 Hz, CH_2CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3256 (υ_{NH}), 3043(υ_{NH}), 1652($\upsilon_{C=O}$), 1242 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 462.4 $[M+1]^+$, 484.4 $[M+Na]^+$; $C_{22}H_{21}ClFN_3O_3S$ (461.1).

2-[(4-nitrophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4b5): Recrystallized from EtOH/DMF as a white crystal, Yield: 26.4%; mp: 224–226 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.75(s, 1H, NH), 10.50 (s, 1H, NH), 8.24 (d, *J*= 9.0 Hz, 2H), 7.74(d, *J*=9.0 Hz, 2H), 7.03–6.93 (m, 3H), 3.97 (s, 2H, *S*-CH_2), 3.81 (s, 2H, CH_2), 2.53 (q, CH_2CH_3, 2H), 1.04 ppm (t, *J*= 7.2 Hz, CH_2CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3301 (υ_{NH}), 3073 (υ_{NH}), 1645 ($\upsilon_{C=O}$), 1249 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 477.1 $[M+1]^+$, 499.1 $[M+Na]^+$; $C_{21}H_{18}ClFN_4O_4S$ (476.07).

2-[(4-cyanophenylamino)carbonylmethylthio]-6-(2-chloro-6-fluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4b6): Recrystallized from EtOH/DMF as a white crystal, Yield: 26.7%; mp: 195–197 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.77 (s, 1H, NH), 10.35(s, 1H, NH), 7.79–6.94 (m, 7H), 4.04 (s, 2H, *S*-CH_2), 3.85 (s, 2H, CH_2), 2.57 (q, *J*= 7.2 Hz, CH_2CH_3, 2H), 1.07 ppm (t, *J*= 7.2 Hz, CH_2CH_3, 3H); ^{13}C NMR (150 MHz, [D$_6$]DMSO): δ= 12.9 (CH_3), 18.2 (CH_2), 34.9 (CH_2-Ph), 37.5 (SCH_2), 109.8 (1C, Ph),114.1 (1C, Ph), 116.7 (C-5),119.1(C≡N), 120.4 (2C, Ph),123.6 (1C, Ph), 126.1(1C, Ph), 128.6 (1C, Ph),131.9 (2C, Ph), 136.6 (1C, Ph), 143.5 (1C, Ph),151.6 (C-6), 160.4 (C-2), 164.6 (C-4), 165.5 (1C, Ph), 169.3 ppm (C=O); IR (KBr): $\tilde{\nu}$= 3347 (υ_{NH}), 3045 (υ_{NH}), 2225 ($\upsilon_{C\equiv N}$), 1640($\upsilon_{C=O}$), 1249 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 457.5 $[M+1]^+$, 479.4 $[M+Na]^+$; $C_{22}H_{18}ClFN_4O_2S$ (456.08).

2-[(4-fluorophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4c1): Recrystallized from EtOH/DMF as a white crystal, Yield: 24.9%; mp: 246–248 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.75 (s, 1H, NH), 10.01 (s, 1H, NH), 7.79 (d, *J*= 9 Hz, 2H), 7.68 (d, *J*= 9 Hz, 2H), 7.08–6.79 (m, 3H), 3.85 (s, 2H, *S*-CH_2), 3.83 (s, 2H, CH_2), 2.00 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3276 (υ_{NH}), 3050 (υ_{NH}), 1672 ($\upsilon_{C=O}$), 1651 ($\upsilon_{C=O}$), 1265 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 420.3 $[M+1]^+$; $C_{20}H_{16}F_3N_3O_2S$ (419.09).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4c2): Recrystallized from EtOH/DMF as a white crystal, Yield: 27.1%; mp: 248–250 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.86 (s, 1H, NH), 10.15 (s, 1H, NH), 7.54 (d, *J*= 9 Hz, 2H), 7.37 (d, *J*= 9 Hz, 2H), 7.15–6.85 (m, 3H), 3.85 (s, 2H, *S*-CH_2), 3.80 (s, 2H, CH_2), 2.00 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3262 (υ_{NH}), 3044 (υ_{NH}), 1658 ($\upsilon_{C=O}$), 1267 (υ_{C-N}), 1247 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 436.4 $[M+1]^+$; $C_{20}H_{16}ClF_2N_3O_2S$ (435.06).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4c3): Recrystallized from EtOH/DMF as a white crystal, Yield: 26.6%; mp: 251–253 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.72 (s, 1H, NH), 10.10 (s, 1H, NH), 7.50–6.87 (m, 7H), 3.85 (s, 2H, *S*-CH_2), 3.82 (s, 2H, CH_2), 2.00 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3261 (υ_{NH}), 3041(υ_{NH}), 16594($\upsilon_{C=O}$), 1267 (υ_{C-N}), 1247 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 480.2 $[M+1]^+$, 502.2 $[M+Na]^+$; $C_{20}H_{16}BrF_2N_3O_2S$ (479.01).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4c4): Recrystallized from EtOH/DMF as a white crystal, Yield: 23.5%; mp: 233–235 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.74 (s, 1H, NH), 9.83 (s, 1H, NH), 7.42–6.87 (m, 7H), 3.87 (s, 2H, *S*-CH_2), 3.77 (s, 2H, CH_2), 3.60 (s, OCH_3, 3H), 2.00 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3257 (υ_{NH}), 3050 (υ_{NH}), 1656 ($\upsilon_{C=O}$), 1248 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 432.4 $[M+1]^+$, 454.3 $[M+Na]^+$; $C_{21}H_{19}F_2N_3O_3S$ (431.11).

2-[(4-nitrophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4c5): Recrystallized from EtOH/DMF as a white crystal, Yield: 25.6%; mp: 258–260 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.77 (s, 1H, NH), 10.58 (s, 1H, NH), 8.24 (d, *J*= 9 Hz, 2H), 7.74 (d, *J*= 9 Hz, 2H), 7.05–6.79 (m, 3H), 3.88 (s, 2H, *S*-CH_2), 3.83 (s, 2H, CH_2), 2.00 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3271 (υ_{NH}), 3055 (υ_{NH}), 1651 ($\upsilon_{C=O}$), 1285 (υ_{C-N}), 1251 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 447.3 $[M+1]^+$; $C_{20}H_{16}F_2N_4O_4S$ (446.09).

2-[(4-cyanophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-methylpyrimidin-4(3*H*)-one (4c6): Recrystallized from EtOH/DMF as a white crystal, Yield: 27.9%; mp: 252–254 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.87 (s, 1H, NH), 10.44 (s, 1H, NH), 7.52–6.89 (m, 7H), 3.85 (s, 2H, *S*-CH_2), 3.80 (s, 2H, CH_2), 2.00 ppm (s, CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3288 (υ_{NH}), 3041(υ_{NH}), 1675 ($\upsilon_{C=O}$), 1654 ($\upsilon_{C=O}$), 2224 ($\upsilon_{C\equiv N}$), 1261 (υ_{C-N}), 1251 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 427.3 $[M+1]^+$; $C_{21}H_{16}F_2N_4O_2S$ (426.1).

2-[(4-fluorophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4d1): Recrystallized from EtOH/DMF as a white crystal, Yield: 23.7%; mp: 225–227 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ= 12.67(s, 1H, NH), 10.02(s, 1H, NH), 7.52–6.87 (m, 7H), 3.88 (s, 2H, *S*-CH_2), 3.79 (s, 2H, CH_2), 2.52 (q, *J*= 7.2 Hz, CH_2CH_3, 2H), 1.01 ppm (t, *J*= 7.2 Hz, CH_2CH_3, 3H); IR (KBr): $\tilde{\nu}$= 3288 (υ_{NH}), 3053 (υ_{NH}), 1672 ($\upsilon_{C=O}$), 1648 ($\upsilon_{C=O}$), 1251 (υ_{C-N}), 1214 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 434.4 $[M+1]^+$; $C_{21}H_{18}F_3N_3O_2S$ (433.11).

2-[(4-chlorophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4d2): Recrystallized from EtOH/DMF as a white crystal, Yield: 26.6%; mp: 222–224 °C (dec);

^{1}H NMR ([D$_6$]DMSO): δ = 12.73 (s, 1H, NH), 10.10 (s, 1H, NH), 7.54 (d, J = 9 Hz, 2H), 7.37 (d, J = 9 Hz, 2H), 7.10–6.85 (m, 3H), 3.86 (s, 2H, *S*-CH$_2$), 3.80 (s, 2H, CH$_2$), 2.51 (q, J = 7.2 Hz, CH$_2$CH$_3$, 2H), 1.01 ppm (t, J = 7.2 Hz, CH$_2$CH$_3$, 3H); ^{13}C NMR (150 MHz, [D$_6$]DMSO): δ = 13.1 (CH$_3$), 18.4 (CH$_2$), 34.1 (CH$_2$-Ph), 37.7 (SCH$_2$), 112.1 (2C, Ph), 112.6 (1C, Ph), 116.3 (C-5), 120.7 (2C, Ph), 127.1(1C, Ph),128.9 (2C, Ph), 132.8 (1C, Ph), 137.6 (1C, Ph), 152.1 (C-6), 160.2 (C-2), 164.1 (C-4), 165.1 (2C, Ph), 169.9 ppm (C=O); IR (KBr): $\tilde{\nu}$ = 3288 (υ_{NH}), 3054 (υ_{NH}), 1673 ($\upsilon_{C=O}$), 1650 ($\upsilon_{C=O}$), 1269 (υ_{C-N}), 1244 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 450.3 [*M*+1]$^+$; $C_{21}H_{18}ClF_2N_3O_2S$ (449.08).

2-[(4-bromophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4d3): Recrystallized from EtOH/DMF as a white crystal, Yield: 23.1%; mp: 223–225 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 12.70 (s, 1H, NH), 10.09 (s, 1H, NH), 7.50 (d, J = 9 Hz, 2H), 7.47 (d, J = 9 Hz, 2H), 7.10–6.85 (m, 3H), 3.86 (s, 2H, *S*-CH$_2$), 3.80 (s, 2H, CH$_2$), 2.53 (q, J = 7.2 Hz, CH$_2$CH$_3$, 2H), 1.01 ppm (t, J = 7.2 Hz, CH$_2$CH$_3$, 3H); IR (KBr): $\tilde{\nu}$ = 3284 (υ_{NH}), 3056 (υ_{NH}), 1657 ($\upsilon_{C=O}$), 1266 (υ_{C-N}), 1243 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 494.2 [*M*+1]$^+$; $C_{21}H_{18}BrF_2N_3O_2S$ (493.03).

2-[(4-methoxyphenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4d4): Recrystallized from EtOH/DMF as a white crystal, Yield: 27.1%; mp: 232–234 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 12.66(s, 1H, NH), 9.85 (s, 1H, NH), 7.42–6.87 (m, 7H), 3.89 (s, 2H, *S*-CH$_2$), 3.76 (s, 2H, CH$_2$), 3.72(s, OCH$_3$, 3H), 2.51 (q, J = 7.2 Hz, CH$_2$CH$_3$, 2H), 1.02 ppm (t, J = 7.2 Hz, CH$_2$CH$_3$, 3H); IR (KBr): $\tilde{\nu}$ = 3250 (υ_{NH}), 3050 (υ_{NH}), 1656 ($\upsilon_{C=O}$), 1241 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 446.3 [*M*+1]$^+$, 468.3 [*M*+Na]$^+$; $C_{22}H_{21}F_2N_3O_3S$ (445.13).

2-[(4-nitrophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4d5): Recrystallized from EtOH/DMF as a white crystal, Yield: 23.3%; mp: 253–255 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 12.76 (s, 1H, NH), 10.57 (s, 1H, NH), 8.24–6.80 (m, 7H), 3.87 (s, 2H, *S*-CH$_2$), 3.85 (s, 2H, CH$_2$), 2.52 (q, J = 7.2 Hz, CH$_2$CH$_3$, 2H), 1.02 ppm (t, J = 7.2 Hz, CH$_2$CH$_3$, 3H); IR (KBr): $\tilde{\nu}$ = 3271 (υ_{NH}), 3056 (υ_{NH}), 1651 ($\upsilon_{C=O}$), 1265 (υ_{C-N}), 1251 (υ_{C-N}); ESI-MS: *m/z* 461.4 [*M*+1]$^+$, 483.3 [*M*+Na]$^+$; $C_{21}H_{18}F_2N_4O_4S$ (460.10).

2-[(4-cyanophenylamino)carbonylmethylthio]-6-(2,6-difluorobenzyl)-5-ethylpyrimidin-4(3*H*)-one (4d6): Recrystallized from EtOH/DMF as a white crystal, Yield: 25.3%; mp: 226–228 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 12.93 (s, 1H, NH), 10.40 (s, 1H, NH), 7.78 (d, J = 9 Hz, 2H), 7.68 (d, J = 9 Hz, 2H), 7.03–6.81 (m, 3H), 3.88 (s, 2H, *S*-CH$_2$), 3.84 (s, 2H, CH$_2$), 2.51 (q, J = 7.2 Hz, CH$_2$CH$_3$, 2H), 1.00 ppm (t, J = 7.2 Hz, CH$_2$CH$_3$, 3H); IR (KBr): $\tilde{\nu}$ = 3288 (υ_{NH}), 3043 (υ_{NH}), 2227 ($\upsilon_{C\equiv N}$), 1646 ($\upsilon_{C=O}$), 1269 (υ_{C-N}), 1249 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 441.4 [*M*+1]$^+$, 463.4 [*M*+Na]$^+$; $C_{22}H_{18}F_2N_4O_2S$ (440.11).

2-[(4-fluorophenylamino)carbonylmethylthio]-6-(1-naphthylmethyl)-5-ethylpyrimidin-4(3*H*)-one (4e1): Recrystallized from EtOH/DMF as a white crystal, Yield: 23.6%; mp: 200–202 °C (dec); ^{1}H NMR ([D$_6$]DMSO): δ = 12.77 (s, 1H, NH), 10.10 (s, 1H, NH), 8.15 (d, J = 8.4 Hz, 1H), 7.89 (d, J = 7.8 Hz, 1H), 7.73 (d, J = 7.8 Hz, 1H), 7.54–7.03 (m, 8H), 4.31 (s, 2H, *S*-CH$_2$), 3.93 (s, 2H, CH$_2$), 2.52 (q, J = 7.2 Hz, CH$_2$CH$_3$, 2H), 0.85 ppm (t, J = 7.2 Hz, CH$_2$CH$_3$, 3H); IR (KBr): $\tilde{\nu}$ = 3262 (υ_{NH}), 3044 (υ_{NH}), 1668 ($\upsilon_{C=O}$), 1649 ($\upsilon_{C=O}$), 1252(υ_{C-N}), 1217 cm^{-1} (υ_{C-N}); ESI-MS: *m/z* 448.2 [*M*+1]$^+$, 470.2 [*M*+Na]$^+$; $C_{25}H_{22}FN_3O_2S$ (447.14)

Anti-HIV activity in MT-4 cells

Evaluation of the antiviral activity of the compounds against HIV-1 strain III$_B$, HIV-2 strain (ROD), and HIV-1 mutant strains in MT-4 cells was performed using the MTT assay as previously described.[16,17] Stock solutions (10× final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock-and HIV-infected cells at the beginning of each experiment. Serial fivefold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA, USA). Untreated control HIV-and mock-infected cell samples were included for each sample.

HIV-1(III$_B$),[20] HIV-2 (ROD),[21] or HIV-1 mutant strains 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 the test compound on uninfected cells to assess the cytotoxicity of the test compound itself. Exponentially growing MT-4 cells[22] were centrifuged for 5 min at 1000 rpm (220 *g*), and the supernatant was discarded. The MT-4 cells were resuspended at 6× 10^5 cells mL^{-1}, and 50 μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by 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 an eight-channel computer-controlled photometer (Multiscan Ascent Reader, Labsystems, Helsinki, Finland), at two wavelengths (540 and 690 nm). All data were calculated using the median OD (optical density) value of three wells. The 50% cytotoxic concentration (CC$_{50}$) was defined as the concentration of the test compound that reduced the absorbance (OD$_{540}$) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus-infected cells was defined as the 50% effective concentration (EC$_{50}$).

RT inhibition assay

Compounds were tested for antiviral activity against HIV-1 RT$_{wt}$, 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 via its 50-terminal phosphate to Covalink-NH microtiter plates. The biotin-dUTP was incorporated by reverse transcriptase. The reaction mixture contained 50 mM Tris-HCl (pH 8.3), 3 mM MgCl$_2$, 75 mM KCl, 5 mM DTT (D,L-dithiothreitol), 0.13 mg mL^{-1} BSA, 10 μg mL^{-1} poly (A), 0.75 μM biotin-11-dUTP, and 1.5 μM dTTP. After incubation at 37 °C for 1 h, the plate was washed three times with a wash buffer containing 50 mM Tris-HCl (pH7.5), 0.15 M NaCl, 0.05 mM MgCl$_2$, and 0.02% Tween-20. After 100 μL of 1% BSA was added to each well and incubated for 30 min at room temperature, the plate was washed with the same buffer. Subsequently 50 μL of SA-ALP (alkaline phosphatase streptavidin) solution (100 ng mL^{-1}) was added per well and then incubated for 1 h at 37 °C. The plate was washed as above and then 50 μL of PNPP (*p*-nitrophenyl phosphate, disodium) (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.

Acknowledgements

Financial support from the Graduate Independent Innovation Foundation of Shandong University (GIIFSDU No. 21310070613090), the Joint Research Fund for Overseas Chinese Young Scholars of the Natural Science Foundation of China (NSFC No. 30928032), the National Natural Science Foundation of China (NSFC Nos. 30873133, 30772629, and 30371686), the Key Project of NSFC for International Cooperation (No. 30910103908), and the Research Fund for the Doctoral Program of Higher Education of China (No. 20070422083) are gratefully acknowledged.

Keywords: antiviral agents · DABOs · HIV-1 · medicinal chemistry · NNRTIs

[1] Uniting the world against AIDS: *AIDS Epidemic Update 2009*, to be found under http://www.unaids.org/en/ (accessed January 18, 2011).
[2] E. De Clercq, *Int. J. Antimicrob. Agents* **2009**, *33*, 307 – 320.
[3] M. P. de Béthune, *Antiviral Res.* **2010**, *85*, 75 – 90.
[4] E. De Clercq, *Antiviral Res.* **2010**, *85*, 19 – 24.
[5] D. Jochmans, *Virus Res.* **2008**, *134*, 171 – 185.
[6] C. Mugnaini, M. Alongi, A. Togninelli, H. Gevariya, A. Brizzi, F. Manetti, C. Bernardini, L. Angeli, A. Tafi, L. Bellucci, F. Corelli, S. Massa, G. Maga, A. Samuele, M. Facchini, I. Clotet-Codina, M. Armand-Ugón, J. A. Esté, M. Botta, *J. Med. Chem.* **2007**, *50*, 6580 – 6595.
[7] M. Artico, S. Massa, A. Mai, M. E. Marongiu, G. Piras, E. Tramontano, P. La Colla, *Antiviral Chem. Chemother.* **1993**, *4*, 361 – 368.
[8] O. J. D'Cruz, F. M. Uckun, *J. Enzyme Inhib. Med. Chem.* **2006**, *21*, 329 – 350.
[9] M. Artico, *Drugs Future* **2002**, *27*, 159 – 175.
[10] M. B. Nawrozkij, D. Rotili, D. Tarantino, G. Botta, A. S. Eremiychuk, I. Musmuca, R. Ragno, A. Samuele, S. Zanoli, M. Armand-Ugón, I. Clotet-Codina, I. A. Novakov, B. S. Orlinson, G. Maga, J. A. Esté, M. Artico, A. Mai, *J. Med. Chem.* **2008**, *51*, 4641 – 4652.
[11] A. Mai, M. Artico, D. Rotili, D. Tarantino, I. Clotet-Codina, M. Armand-Ugón, R. Ragno, S. Simeoni, G. Sbardella, M. B. Nawrozkij, A. Samuele, G. Maga, J. A. Esté, *J. Med. Chem.* **2007**, *50*, 5412 – 5424.
[12] M. Yu, X. Liu, Z. Li, S. Liu, C. Pannecouque, E. De Clercq, *Bioorg. Med. Chem.* **2009**, *17*, 7749 – 7754.
[13] S. M. Hannick, Y. Kishi, *J. Org. Chem.* **1983**, *48*, 3833 – 3835.
[14] K. Danel, E. Larsen, E. B. Pedersen, *Synthesis* **1995**, 934 – 936.
[15] M. Radi, C. Falciani, L. Contemori, E. Petricci, G. Maga, A. Samuele, S. Zanoli, M. Terrazas, M. Castria, A. Togninelli, J. A. Esté, I. Clotet-Codina, M. Armand-Ugón, M. Botta, *ChemMedChem* **2008**, *3*, 573 – 593.
[16] R. Pauwels, J. Balzarini, M. Baba, R. Snoeck, D. Schols, P. Herdewijn, J. Desmyter, E. De Clercq, *J. Virol. Methods* **1988**, *20*, 309 – 321.
[17] C. Pannecouque, D. Daelemans, E. De Clercq, *Nat. Protoc.* **2008**, *3*, 427 – 434.
[18] A. L. Hopkins, J. Ren, R. M. Esnouf, B. E. Willcox, E. Y. Jones, C. Ross, T. Miyasaka, R. T. Walker, H. Tanaka, D. K. Stammers, D. I. Stuart, *J. Med. Chem.* **1996**, *39*, 1589 – 1600.
[19] J. Ren, C. Nichols, L. Bird, P. Chamberlain, K. Weaver, S. Short, D. I. Stuart, D. K. Stammers, *J. Mol. Biol.* **2001**, *312*, 795 – 805.
[20] M. Popovic, M. G. Sarngadharan, E. Read, R. C. Gallo, *Science* **1984**, *224*, 497 – 500.
[21] F. Barre-Sinoussi, J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Grest, C. Dauget, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, L. Montagnier, *Science* **1983**, *220*, 868 – 871.
[22] I. Miyoshi, H. Taguchi, I. Kobonishi, S. Yoshimoto, Y. Ohtsuki, Y. Shiraishi, T. Akagi, *Gann Monogr.* **1982**, *28*, 219 – 228.

Received: December 28, 2010
Revised: January 5, 2011
Published online on February 14, 2011

