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5-Alkyl-2-alkylamino-6-(2,6-difluorophenylalkyl)-3,4-dihydropyrimidin-4(3*H*)-ones, a new series of potent, broad-spectrum non-nucleoside reverse transcriptase inhibitors belonging to the DABO family

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Abstract—2-Alkylamino-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-ones (F_2-*NH*-DABOs) **4**, **5** belonging to the dihydro-alkoxy-benzyl-oxopyrimidine (DABO) family and bearing different alkyl- and arylamino side chains at the C_2-position of the pyrimidine ring were designed as active against wild type (wt) human immunodeficiency virus type 1 (HIV-1) and some relevant HIV-1 mutants. Biological evaluation indicated the importance of the further anchor point of compounds **4**, **5** into the non-nucleoside binding site (NNBS): newly synthesized compounds were highly active against both wild type and the Y181C HIV-1 strains. In anti-wt HIV-1 assay the potency of amino derivatives did not depend on the size or shape of the C_2-amino side chain, but it associated with the presence of one or two methyl groups (one at the pyrimidine C_5-position and the other at the benzylic carbon), being thymine, α-methyluracil or α-methylthymine derivatives almost equally active in reducing wt HIV-1-induced cytopathogenicity in MT-4 cells. Against the Y181C mutant strain, 2,6-difluorobenzyl-α-methylthymine derivatives **4d**, **5h′**–**n′** showed the highest potency and selectivity among tested compounds, both a properly sized C_2–NH side chain and the presence of two methyl groups (at C_5 and benzylic positions) being crucial for high antiviral action.

1. Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are useful drugs for the treatment of human immunodeficiency virus type 1 (HIV-1) infections when used in combination with other anti-HIV agents such as nucleoside analogs (NRTIs) and protease inhibitors (PIs).[1–4]

First generation NNRTIs include more than 30 classes of compounds. Among them, nevirapine[5,6] (*Viramune*, Boehringer Ingelheim) was the first product approved in 1996 as anti-AIDS drug by the US Food and Drug Administration (FDA), followed in 1997 by delavirdine

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mesylate[7,8] (*Rescriptor*, Pharmacia & Upjohn) (Fig. 1). These inhibitors of HIV-1 reverse transcriptase (RT) show low toxicity and favorable pharmacokinetic properties, but they suffer from the rapid selection for resistant viral populations both in cell cultures and patients.[6,9–11] Mutations commonly selected for NNRTIs occur at 98–108, 179–190, and 230–236 amino acid positions, with Y181C, K103N or both being the most clinically relevant. Therefore, in the NNRTI field the efforts are now focused on the development of new compounds endowed with higher anti-HIV-1 activity and a resistance profile different from that of known drugs (second generation NNRTIs).

Efavirenz (*Sustiva*, DuPont)[12] has been approved by FDA in 1998 for the therapy of HIV-1 infections in combination with other anti-HIV drugs, and the imidazole derivative capravirine (AG1549, Pfizer)[13] is under Phase III clinical trials. Clinical development of emivirine, a HEPT derivative formerly known as MKC-442,[14,15] has been halted by Triangle Pharmaceuticals in January 2002 when a comparative study showed emivirine to be less potent than other antiretrovirals (Fig. 1).

Dihydro-alkoxy-benzyl-oxopyrimidines (DABOs) were reported in 1992 as a novel NNRTI class.[16–18] Since then, a number of oxopyrimidines were synthesized and tested as anti-HIV-1 agents with the aim to obtain more potent and selective compounds.[19–28] Structure–activity relationship (SAR) profile of DABOs together with molecular modeling investigation on their putative binding mode have shown that the presence of a C_2-alkoxy (DABOs, **1**) or C_2-alkylthio (*S*-DABOs, **2**) side chain is a structural determinant for the antiviral activity of these derivatives, with the length and size of the C_2 side chain having only modulator effects on potency.[19–22] Moreover, the NHCO fragment at N_3/C_4-positions of pyrimidine ring, stabilized by a hydrogen bond between the 3-NH function of *S*-DABOs and the carbonyl oxygen of Lys101, cannot be modified without abolishing the antiviral activity of DABO derivatives,[24] and the 2,6-difluoro substitution at the C_6 phenylmethyl moiety of *S*-DABOs produces favorable π-stacking interactions with the Tyr188 side chain and leads to compounds active in the nanomolar range (difluoro-thio-DABOs, F_2-*S*-DABOs **3**, i.e., **3a**,**b**).[25] Finally, the presence in the *S*-DABO skeleton of two methyl groups, the former at the pyrimidine C_5-position and the latter on the methylene bridge between pyrimidine and phenyl rings (benzylic position), furnished new conformationally restricted F_2-*S*-DABOs **3** (i.e., **3c**,**d**) exhibiting potent activity in the low nanomolar range against wild-type HIV-1 and at submicromolar concentrations against the Y181C mutant strain of HIV-1 (Fig. 1).[27] 2-Cyclopentylthio-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methylpyrimidin-4(3*H*)-one **3d** turned out the most potent and selective among the *S*-DABOs reported to

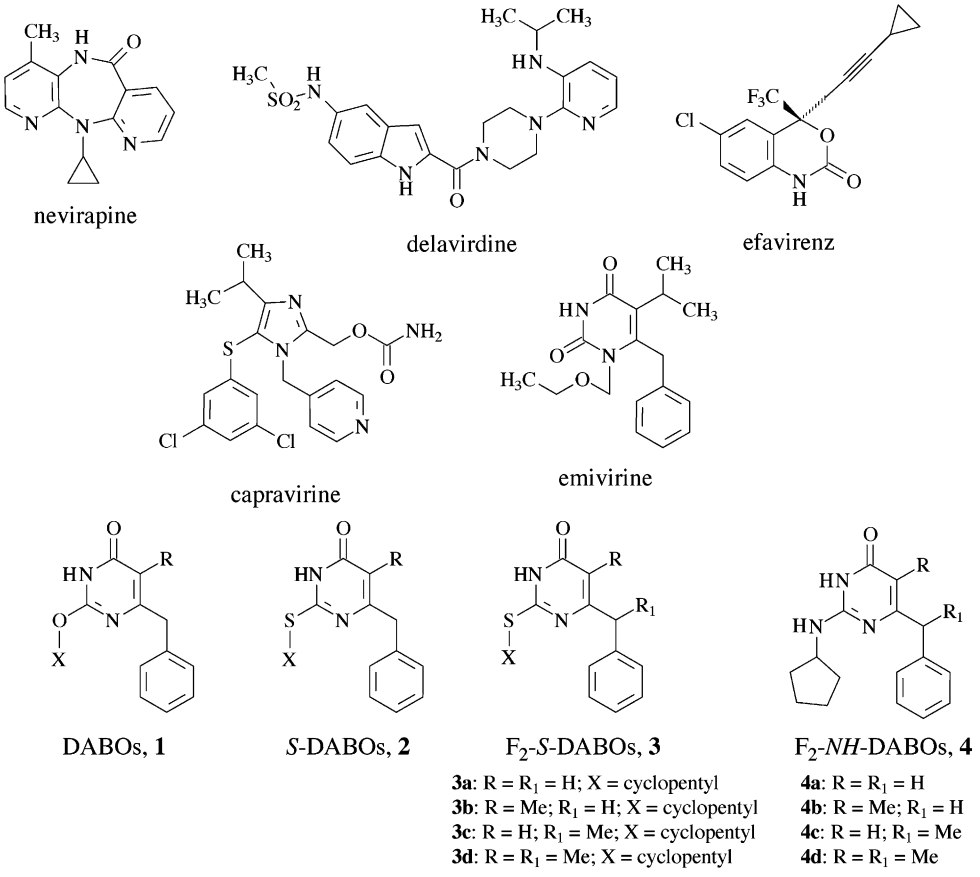


Figure 1. Structures of known non-nucleoside reverse transcriptase inhibitors.

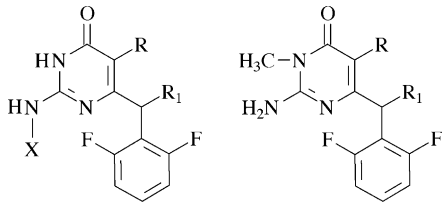
date, with EC_{50} against wt HIV-1 = 6 nM, and EC_{50} against the Y181C HIV-1 mutant = 200 nM.

On the basis of these findings, we made computational studies and structure-based design of a novel series of DABOs (compounds **4a**–**d**, difluoro-amino-DABOs, F_2-*NH*-DABOs), characterized by the replacement of the C_2-cyclopentylthio moiety of **3d** with a C_2-cyclopentylamino side chain, beside the hydrogen or methyl substitutions at the C_5 and/or at the benzylic carbon, and the highly favorable 2,6-difluorophenylmethyl moiety at C_6-position of the pyrimidine ring (Fig. 1).[28] The aim of such modification was to introduce structural features in the DABO skeleton able to give more interaction points with the HIV-1 RT non-nucleoside binding site (NNBS), that is, through a hydrogen bond formed between the novel C_2–NH function and the enzyme backbone, necessary for a tighter binding with RT.

As **4a**–**d** were highly active in both anti-RT and anti-HIV biological assays, and 2-cyclopentylamino-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methylpyrimidin-4(3*H*)-one **4d** showed an EC_{50} against Y181C HIV-1 mutant variant = 160 nM with the fold resistance value (5.3) 2-fold lower than that of its 2-cyclopentylthio counterpart **3d** (13),[28] we prepared a large series of 2-alkylamino-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-one derivatives **5a**–**n′**, bearing differently sized and shaped alkyl- and arylamino side chains at the C_2-position of the pyrimidine ring, to be tested as anti-HIV-1 agents (Fig. 2). The inhibitory effects against wild type HIV-1 rRT and a panel of mutant RTs (K103N, L100I, V106A, V179D, Y181I, and Y188L) were determined on **3d** and **4d**, as representative members of F_2-*S*- and F_2-*NH*-DABOs, respectively, and the activity of selected **4**, **5** compounds were assessed against Y181C HIV-1 mutant strain in comparison with those of F_2-*S*-DABOs **3a**–**d**.

In parallel with the synthetic effort, we performed docking analysis of **3d** and **4d** into the NNBS of both wt and Y181C HIV-1 RT, in comparison with TNK-651,[15,29] a HEPT analog, which was co-crystallized in wt as well as Y181C HIV RT enzymes.

Difluoro-amino-DABOs **6**–**10** bearing at C_2–NH-position a hydrogen (**6**, **7**) or chemical functions different from alkyl or aryl chains (**8**–**10**) (Fig. 2) were also prepared and tested to gain further SAR information.



5a-n',7-10 **6a-c**

5a-n': R = R_1 = H, Me; X = alkyl, aryl, arylalkyl
7: R = R_1 = X = H
8: R = R_1 = H, Me; X = CN
9: R = R_1 = H, Me; X = NO_2
10: R = R_1 = H; X = $NHCOCH_2CH_3$

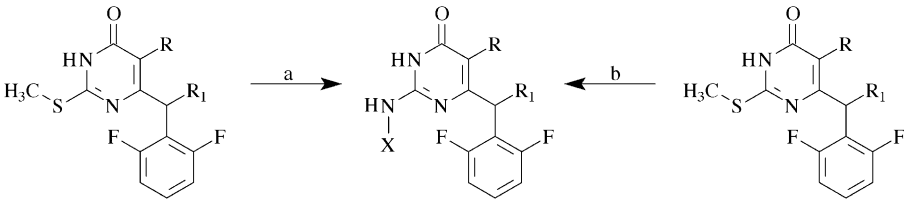
R = R_1 = H, Me

Figure 2. New designed amino-DABO derivatives.

2. Chemistry

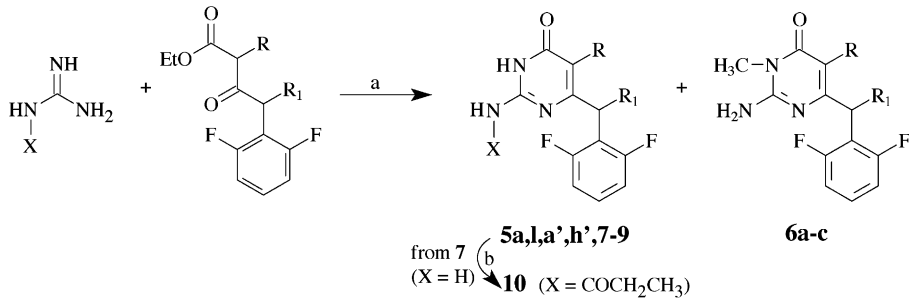
Most of difluoro-amino-DABOs **5a**–**n′** were obtained from 5-alkyl-6-(2,6-difluorophenylalkyl)-3,4-dihydro-2-methylthiopyrimidin-4(3*H*)-ones[25,27] by nucleophilic displacement of the C_2-methylthio group of the pyrimidine ring with the appropriate primary amine. Two different procedures have been used to achieve methylthio displacement, depending on the amine boiling points. With highly boiling amines (bp >60 °C) the reaction was carried out in a sealed tube heating the starting material in the presence of an excess of the amine at the temperature of 130 °C (anilines) or 170 °C (alkyl- and arylalkylamines) (Scheme 1). With amine having a boiling point of <60 °C, the reaction was performed in oil bath at 180 °C by mixing the 2-methylthioderivative with the amine as acetate salt (Scheme 1). 2-Methylamino derivatives **5a**,**l**,**a′**,**h′** and their by-products 2-amino-3-methylpyrimidin-4(3*H*)-ones **6a**–**c**, 2-aminopyrimidin-4(3*H*)-one **7**, and 2-cyanoamino- and 2-nitroaminopyrimidin-4(3*H*)-one derivatives **8** and **9** have been prepared through condensation of the appropriate ethyl 2,6-difluorophenylacetylacetates with methylguanidine, guanidine, cyanoguanidine, or nitroguanidine in alkaline medium (Scheme 2). Acylation of **7** with propionyl chloride under standard conditions furnished the 2-propionamide-4(3*H*)-pyrimidinone **10** (Scheme 2).

Chemical and physical data for derivatives **5**–**10** are reported in Table 1.



5b-k,m-z,b'-g',i'-n'

Scheme 1. Reagents and conditions: (a) X–NH_2, 130–170 °C, sealed tube (compounds **5f**–**k**,**o**–**z**,**d′**–**g′**,**k′**–**n′**); (b) CH_3COONH_3–X, 180 °C, oil bath (compounds **5b**–**e**,**m**,**n**,**b′**,**c′**,**i′**,**j′**). R = R_1 = H, Me; X = Et, *n*-Pr, *i*-Pr, cyclopropyl, *n*-Bu, *s*-Bu, methoxyethyl, methylthioethyl, cyclohexyl, (substituted)phenyl, phenylmethyl, 1-naphthyl.



Scheme 2. Reagents and conditions: (a) EtONa, EtOH, reflux; (b) propionyl chloride, Et_3N, THF, rt. R = R_1 = H, Me; X = H, Me, CN, NO_2, $COCH_2CH_3$.

3. Results and discussion

3.1. Cytotoxicity, anti-HIV-1 and anti-RT activities, and SAR studies

Title compounds **5a**–**n**′ are characterized by the presence of differently shaped alkylamino or arylamino side chains at C_2-position of the pyrimidine, and they can be divided into four groups: (i) 6-(2,6-difluorophenylmethyl)uracils **5a**–**k**, (ii) 6-(2,6-difluorophenylmethyl)-thymines **5l**–**z**, and (iii) and (iv) 6-[1-(2,6-difluorophenyl)ethyl] analogs of both the uracil and thymine series **5a**′–**g**′ and **5h**′–**n**′, respectively.

All designed compounds were evaluated for cytotoxicity and anti-HIV-1 activity in MT-4 cells, in comparison with nevirapine and emivirine used as reference drugs (Table 2).

Most of compounds **5a**–**z** were not cytotoxic for MT-4 cells at doses as high as 200 μM, and only six of them (**5j**,**k**,**q**,**r**,**t**,**z**) showed CC_{50} values at concentrations lower than 100 μM. Introduction of a methyl group at the benzylic position of F_2-*NH*-DABOs (compounds **5a**′–**n**′) led to more cytotoxic derivatives, both in the uracil and thymine related series, with CC_{50} values ranging from 44 to 115 μM. Exceptions to this rule were **5h**′,**j**′, and **n**′, which resulted less cytotoxic (CC_{50} values $\geqslant$200 μM).

In previous DABO series[19–22,24–27] the maximum anti-HIV-1 potency correlated with the size of the C_2-alkylthio side chain rather than with the nature of the base (uracil or thymine), whereas in the F_2-*NH*-DABOs the antiviral activity resulted slightly or not dependent on the size of the C_2-alkyl/arylamino substituent. In fact, compounds bearing 2-alkyl/arylamino chains with different length and size showed very similar EC_{50} values. Exceptions to this rule are the C_2-methylamino and -ethylamino derivatives **5a**,**b**,**l**,**a**′, which having a small C_2 substituent showed a decrease of anti-HIV-1 activity, and the cyclopropylamino derivative **5e**, 8- and 29-fold less potent than the *n*-propylamino and *i*-propylamino counterparts **5c** and **5d**, respectively. The 2-methoxyethylamino compound **5h** resulted 8 times less potent than the corresponding 2-*n*-butylamino analog **5f**, showing that the introduction of lipophilic side chain was preferred at C_2-position.

In spite of this small difference in potency among compounds carrying different alkyl/arylamino chains, the introduction of a C_5-methyl group in the uracil base furnished more potent and selective derivatives (compare **5l**–**s** with the corresponding C_5–H analogs). Less evidently, this rule was confirmed by the EC_{50} values shown by the 6-[1-(2,6-difluorophenyl)ethyl] analogs **5a**′–**g**′, and **5h**′–**n**′, in which the thymine compounds resulted 2–3 times more active than the uracil counterparts.

The introduction of a methyl substituent in the benzylic carbon of F_2-*NH*-DABOs in the uracil series (group (iii)) gave compounds showing EC_{50} values from 3 to 14 times lower than the corresponding values of unsubstituted derivatives (compare **5a**′–**g**′ with their benzylic-unsubstituted analogs). Differently, in the thymine series (group (iv)) this substitution did not influence the antiviral activity, with the exception of the 2-methylamino compound **5h**′, which resulted 13 times more active than the unsubstituted analog **5l**. Therefore, while in previously reported F_2-*S*-DABOs the methylation of the benzylic carbon improved anti-HIV-1 inhibitory activity together with selectivity, in F_2-*NH*-DABOs the double C_5 and benzylic methyl substitution did not produce increase of antiviral activity.

Comparing F_2-*NH*-DABOs with the reference drugs, most of DABO derivatives sharing C_5 and/or benzylic methyl substituents resulted 8- to 10-fold more potent than nevirapine and endowed with the same potency of MKC-442. Moreover, two compounds (**5i**′ and **5j**′) were 3- to 6-fold more potent and three (**5m**,**o**, and **5b**) were more selective than MKC-442.

Representative F_2-*NH*-DABOs were tested in enzyme assays against highly purified HIV-1 rRT using poly(rC)–oligo$(dG)_{12–18}$ as template primer, and an excellent correlation between the IC_{50} (enzyme inhibiting activity) and EC_{50} (in vitro antiproliferative effect) values was found (Table 3). Moreover, the F_2-*S*-DABO and the F_2-*NH*-DABO prototypes **3d** and **4d** were assayed against a panel of rRTs containing mutations (K103N, L100I, V106A, V179D, Y181I, and Y188L) known to confer resistance to NNRTIs (Table 4). Despite **4d** being less potent than **3d** against wt RT, data reported in Table 4 clearly show for **4d** a better fold-resistance profile against mutant RTs than that for **3d**.

Table 1. Physical and chemical data of compounds **5**–**10**

Compound	R	R_1	X	Mp (°C)	Recryst solvent	Synth scheme (method)[a]	% Yield	Formula[b]
5a	H	H	Methyl	202–203	Acetonitrile	2	58	$C_{12}H_{11}F_2N_3O$
5b	H	H	Ethyl	138–140	*n*-Hexane/cyclohexane	1 (b)	60	$C_{13}H_{13}F_2N_3O$
5c	H	H	*n*-Propyl	136–137	Cyclohexane	1 (b)	44	$C_{14}H_{15}F_2N_3O$
5d	H	H	*iso*-Propyl	150–151	Diethyl ether	1 (b)	52	$C_{14}H_{15}F_2N_3O$
5e	H	H	Cyclopropyl	183–184	Benzene/cyclohexane	1 (b)	58	$C_{14}H_{13}F_2N_3O$
5f	H	H	*n*-Butyl	130–131	*n*-Hexane	1 (a)	65	$C_{15}H_{17}F_2N_3O$
5g	H	H	*sec*-Butyl	140–141	Diethyl ether	1 (a)	57	$C_{15}H_{17}F_2N_3O$
5h	H	H	Methoxyethyl	120–121	Acetonitrile	1 (a)	68	$C_{14}H_{15}F_2N_3O_2$
5i	H	H	Methylthioethyl	128.5–129	Acetonitrile	1 (a)	59	$C_{14}H_{15}F_2N_3OS$
5j	H	H	Cyclohexyl	143–144	Diethyl ether	1 (a)	60	$C_{17}H_{19}F_2N_3O$
5k	H	H	Phenyl	223–224	Acetonitrile	1 (a)	68	$C_{17}H_{13}F_2N_3O$
5l	Me	H	Methyl	210–211	Acetonitrile	2	52	$C_{13}H_{13}F_2N_3O$
5m	Me	H	*n*-Propyl	156–157	Acetonitrile	1 (b)	46	$C_{15}H_{17}F_2N_3O$
5n	Me	H	*iso*-Propyl	165–166	*n*-Hexane	1 (b)	65	$C_{15}H_{17}F_2N_3O$
5o	Me	H	*n*-Butyl	192–193	Acetonitrile	1 (a)	70	$C_{16}H_{19}F_2N_3O$
5p	Me	H	*sec*-Butyl	Oil		1 (a)	51	$C_{16}H_{19}F_2N_3O$
5q	Me	H	Methylthioethyl	150–151	Acetonitrile	1 (a)	55	$C_{15}H_{17}F_2N_3OS$
5r	Me	H	Cyclohexyl	150–151	*n*-Hexane	1 (a)	67	$C_{18}H_{21}F_2N_3O$
5s	Me	H	Phenyl	226–227	Acetonitrile	1 (a)	57	$C_{18}H_{15}F_2N_3O$
5t	Me	H	2,6-F_2-phenyl	214–215	Acetonitrile	1 (a)	55	$C_{18}H_{13}F_4N_3O$
5u	Me	H	2-Cl-phenyl	272–273	Acetonitrile	1 (a)	48	$C_{18}H_{14}ClF_2N_3O$
5v	Me	H	3-Cl-phenyl	231–232	Acetonitrile	1 (a)	58	$C_{18}H_{14}ClF_2N_3O$
5w	Me	H	4-Cl-phenyl	245–246	Acetonitrile	1 (a)	64	$C_{18}H_{14}ClF_2N_3O$
5x	Me	H	4-Me-phenyl	235–236	Acetonitrile	1 (a)	59	$C_{19}H_{17}F_2N_3O$
5y	Me	H	1-Naphthyl	>280	Acetonitrile	1 (a)	47	$C_{22}H_{17}F_2N_3O$
5z	Me	H	Phenylmethyl	182–183	Acetonitrile	1 (a)	56	$C_{19}H_{17}F_2N_3O$
5a′	H	Me	Methyl	145–146	Cyclohexane	2	49	$C_{13}H_{13}F_2N_3O$
5b′	H	Me	*n*-Propyl	Oil		1 (b)	69	$C_{15}H_{17}F_2N_3O$
5c′	H	Me	*iso*-Propyl	Oil		1 (b)	55	$C_{15}H_{17}F_2N_3O$
5d′	H	Me	*n*-Butyl	Oil		1 (a)	58	$C_{16}H_{19}F_2N_3O$
5e′	H	Me	*sec*-Butyl	Oil		1 (a)	62	$C_{16}H_{19}F_2N_3O$
5f′	H	Me	Methylthioethyl	113–114	Diethyl ether	1 (a)	52	$C_{15}H_{17}F_2N_3OS$
5g′	H	Me	Cyclohexyl	Oil		1 (a)	48	$C_{18}H_{21}F_2N_3O$
5h′	Me	Me	Methyl	193–194	Benzene/cyclohexane	2	55	$C_{14}H_{15}F_2N_3O$
5i′	Me	Me	*n*-Propyl	Oil		1 (b)	58	$C_{16}H_{19}F_2N_3O$
5j′	Me	Me	*iso*-Propyl	157–157.5	Diethyl ether	1 (b)	67	$C_{16}H_{19}F_2N_3O$
5k′	Me	Me	*n*-Butyl	Oil		1 (a)	52	$C_{17}H_{21}F_2N_3O$
5l′	Me	Me	Methylthioethyl	106–107	Diethyl ether	1 (a)	44	$C_{16}H_{19}F_2N_3OS$
5m′	Me	Me	Cyclohexyl	Oil		1 (a)	59	$C_{19}H_{23}F_2N_3O$
5n′	Me	Me	Phenyl	210–211	Acetonitrile	1 (a)	54	$C_{19}H_{17}F_2N_3O$
6a	H	H		215–216	Acetonitrile	2	18	$C_{12}H_{11}F_2N_3O$
6b	H	Me		197–198	Acetonitrile	2	12	$C_{13}H_{13}F_2N_3O$
6c	Me	Me		197–198	Benzene	2	16	$C_{14}H_{15}F_2N_3O$
7	H	H	H	276.5–277	Ethanol	2	78	$C_{11}H_9F_2N_3O$
8a	H	H	CN	255–256	Ethanol/water	2	86	$C_{12}H_8F_2N_4O$
8b	Me	H	CN	276–277	Ethanol/water	2	79	$C_{13}H_{10}F_2N_4O$
9a	H	H	NO_2	212–214	Ethanol/water	2	85	$C_{11}H_8F_2N_3O_3$
9b	Me	H	NO_2	197–198	Ethanol/water	2	88	$C_{12}H_{10}F_2N_3O_3$
10	H	H	$COCH_2CH_3$	163–163.5	Benzene	2	75	$C_{14}H_{13}F_2N_3O_2$

[a] See Schemes 1 and 2.
[b] All compounds were analyzed for C, H, N, S, F. Analytical results were within ±0.4% of the theoretical values.

Finally, selected F_2-*NH*-DABOs **4**, **5** were tested in cell culture using MT-4 cells and both wild-type virus and virus containing a mutation in residue Tyr181 to Cys, known to be crucial to confer NNRTI resistance in treated patients, in comparison with compounds **3a**–**d** (F_2-*S*-DABOs), nevirapine, and efavirenz (Table 5).

All tested DABO derivatives (with the exception of **5s**) retained their inhibitory activity against the HIV-1 Y181C mutant strain, being their EC_{50} values included in the low micromolar/submicromolar range. In the amino-DABO series, similarly to that observed with F_2-*S*-DABOs,[27] the contemporary presence of two methyl groups both in C_5 and in benzylic position of the pyrimidine ring strongly contributed to maintain the antiviral potency against the Y181C strain in the low submicromolar range. Particularly, **4d** and **5k′** showed EC_{50} values against Y181C mutant only 4–5 times higher than those showed against wild-type HIV-1. The C_2-methylamino derivative **5h′**, belonging to the same series of **4d** and **5k′** (group (iv)), showed a Y181C/wt HIV-1 EC_{50} ratio of 650, showing that beside the double-methyl substitution a bulky C_2 side

Table 2. Cytotoxicity and anti-HIV-1 activity of compounds **5**–**10**[a]

Compound	R	R_1	X	CC_{50}[b] (μM)	EC_{50}[c] (μM)	SI[d]
5a	H	H	Methyl	>200[e]	1.5	>133
5b	H	H	Ethyl	>200	0.8	>250
5c	H	H	*n*-Propyl	200	0.11	1818
5d	H	H	*iso*-Propyl	>200	0.4	>500
5e	H	H	Cyclopropyl	>200	3.17	>63
5f	H	H	*n*-Butyl	100	0.1	1000
5g	H	H	*sec*-Butyl	>200	0.13	>1543
5h	H	H	Methoxyethyl	>200	0.80	>250
5i	H	H	Methylthioethyl	>100	0.3	>333
5j	H	H	Cyclohexyl	66	0.14	476
5k	H	H	Phenyl	60	0.29	207
5l	Me	H	Methyl	135	0.4	335
5m	Me	H	*n*-Propyl	>200	0.02	>10,000
5n	Me	H	*iso*-Propyl	190	0.03	6333
5o	Me	H	*n*-Butyl	>200	0.02	>10,000
5p	Me	H	*sec*-Butyl	200	0.06	3333
5q	Me	H	Methylthioethyl	82	0.016	5125
5r	Me	H	Cyclohexyl	30	0.03	1000
5s	Me	H	Phenyl	>200	0.06	>3333
5t	Me	H	2,6-F_2-phenyl	55.5	0.14	396
5u	Me	H	2-Cl-phenyl	>200	0.13	>1538
5v	Me	H	3-Cl-phenyl	>200	0.2	>1000
5w	Me	H	4-Cl-phenyl	>200	0.1	>2000
5x	Me	H	4-Me-phenyl	>200	0.18	>1111
5y	Me	H	1-Naphthyl	>200	>200	
5z	Me	H	Phenylmethyl	50	0.50	100
5a′	H	Me	Methyl	106	0.11	964
5b′	H	Me	*n*-Propyl	103	0.02	5150
5c′	H	Me	*iso*-Propyl	115	0.03	3830
5d′	H	Me	*n*-Butyl	52	0.03	1730
5e′	H	Me	*sec*-Butyl	86	0.04	2150
5f′	H	Me	Methylthioethyl	44	0.013	3385
5g′	H	Me	Cyclohexyl	56	0.022	2545
5h′	Me	Me	Methyl	200	0.03	6666
5i′	Me	Me	*n*-Propyl	70	0.006	11,666
5j′	Me	Me	*iso*-Propyl	200	0.005	40,000
5k′	Me	Me	*n*-Butyl	83	0.01	8300
5l′	Me	Me	Methylthioethyl	>100	0.14	>714
5m′	Me	Me	Cyclohexyl	58	0.026	2231
5n′	Me	Me	Phenyl	>200	0.024	>8333
6a	H	H		>200	55	>3
6b	H	Me		>200	2.4	>83
6c	Me	Me		93	>93	
7	H	H	H	>200	108	>1.8
8a	H	H	CN	>200	>200	
8b	Me	H	CN	>200	>200	
9a	H	H	NO_2	>200	>200	
9b	Me	H	NO_2	>200	>200	
10	H	H	$COCH_2CH_3$	>200	>200	
4a[f]	H	H	Cyclopentyl	>200	0.09	>2222
4b[f]	Me	H	Cyclopentyl	>200	0.02	>10,000
4c[f]	H	Me	Cyclopentyl	76	0.03	3500
4d[f]	Me	Me	Cyclopentyl	90	0.02	4500
Emivirine				200	0.03	6666
Nevirapine				>200	0.3	>666

[a] Data represent mean values of at least two separate experiments.
[b] Compound dose required to reduce the viability of mock-infected cells by 50%, as determined by the MTT method.
[c] Compound dose required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity, as determined by the MTT method.
[d] Selectivity index, CC_{50}/EC_{50} ratio.
[e] Higher concentrations could not be achieved for crystallization of compounds in the culture medium.
[f] Ref. 28.

chain (at least four carbon atoms) is requested to obtain a high anti-Y181C HIV-1 activity.

Compared with the thio-DABO **3d**, the amino-DABOs **4d** and **5k′** showed a lower fold resistance of antiviral

Table 3. Activity of selected F_2-*NH*-DABOs **4**, **5** against HIV-1 rRT[a]

Compound	R	R_1	X	IC_{50}[b] (μM)
5a	H	H	Methyl	2.5
4a[c]	H	H	Cyclopentyl	0.07
4b[c]	Me	H	Cyclopentyl	0.03
5s	Me	H	Phenyl	0.15
4c[c]	H	Me	Cyclopentyl	0.04
4d[c]	Me	Me	Cyclopentyl	0.035
Emivirine				0.04

[a] Data represent mean values of at least two separate experiments.
[b] Compound dose required to inhibit the HIV-1 rRT activity by 50%.
[c] Ref. 28.

Table 4. Activity of the F_2-*S*-DABO **3d** and the F_2-*NH*-DABO **4d** against a panel of mutant rRTs

HIV-1 RT	K_i Values (μM) (*n*)[a]	
	3d	**4d**
WT	0.005	0.03
K103N	0.03 (6)	0.08 (3)
L100I	0.3 (60)	0.5 (17)
V106A	0.5 (100)	0.5 (17)
V179D	0.3 (60)	0.3 (10)
Y181I	0.9 (180)	1.7 (57)
Y188L	1.4 (280)	1.1 (37)

[a] (*n*): Fold resistance.

Table 5. Activity of selected DABOs against Y181C HIV-1 mutant strain: comparison between F_2-*S*-DABO (**3**) and F_2-*NH*-DABO (**4** and **5**) activities

Compound	R	R_1	X	EC_{50}[a] (μM)		(Y181C/WT_{IIIB})[b]
				WT_{IIIB}	Y181C	
3a	H	H	Cyclopentyl	0.3	5.3	(18)
3b	Me	H	Cyclopentyl	0.04	1.2	(30)
3c	H	Me	Cyclopentyl	0.05	2.5	(50)
3d[c]	Me	Me	Cyclopentyl	0.015	0.2	(13)
4a	H	H	Cyclopentyl	0.2	2.1	(10)
4b	Me	H	Cyclopentyl	0.02	0.8	(42)
4c	H	Me	Cyclopentyl	0.02	0.5	(25)
4d[c]	Me	Me	Cyclopentyl	0.03	0.16	(5.3)
5o	Me	H	*n*-Butyl	0.01	0.7	(70)
5p	Me	H	*sec*-Butyl	0.06	2.3	(38)
5s	Me	H	Phenyl	0.05	>100	
5d′	H	Me	*n*-Butyl	0.03	0.6	(20)
5e′	H	Me	*sec*-Butyl	0.04	1.4	(35)
5h′	Me	Me	Methyl	0.02	13	(650)
5i′	Me	Me	*n*-Propyl	0.01	0.2	(20)
5j′	Me	Me	*iso*-Propyl	0.02	0.5	(29)
5k′	Me	Me	*n*-Butyl	0.04	0.2	(4.5)
Nevirapine				0.37	>30	(>81)
Efavirenz				0.004	0.025	(6)

[a] Compound dose required to achieve 50% protection of MT-4 cells for wild-type and Y181C HIV-1-induced cytopathogenicity, as determined by the MTT method.
[b] Fold resistance.
[c] Ref. 28.

activity from wild type to Y181C HIV-1 strains (**3d** = 10; **4d** = 5.3; **5k′** = 4.5).

3.2. Binding mode investigation of F_2-*NH*-DABOs in wild-type and Y181C mutant forms of HIV-1 RT

In a previous study[28] we found that the possible role of S → NH replacement in the F_2-*S*-DABO series was the introduction of a new anchor point for the binding of F_2-*NH*-DABOs into the NNBS of the HIV-1 RT. Docking studies revealed that a hydrogen bond is formed between the C_2-NH group and the carbonyl oxygen of Lys101 (N–O distance = 2.779 Å). The F_2-*NH*-DABO/RT_{wt} hydrogen bonding pattern resembles that of the DNA base pairs, and such interactions seem to stick the ligand in such a way that in part diminish the consequence of the double methyl substitution at C_5 and benzylic carbon positions of the pyrimidine, which was crucial for the anti-HIV-1 activity of F_2-*S*-DABOs (compare EC_{50} values of **3a–d**[25,27] and **4a–d**[28] against wild type HIV-1). Moreover, an improvement of the anti-HIV activity profile against the Y181C mutant strain was observed for the F_2-*NH*-DABOs **4a–d**.[28]

In view of the above observations, docking studies were performed in parallel on the wild type (RT_{wt}) and the Y181C mutant (RT_{Y181C}) form of the HIV-1 RT. For these studies, **4d** was used as representative member of F_2-*NH*-DABOs and docked into the NNBS of either RT_{wt} or RT_{Y181C}. For comparison purposes, the same docking procedure was carried out on the conformationally constrained F_2-*S*-DABO **3d** and also on TNK-651, a potent HEPT analog, to assess the reliability of the docking procedure.

The coordinates of TNK-651 co-crystallized in either the RT_{wt} (PDB entry code 1rt2)[15] or RT_{Y181C} (PDB entry code 1jla)[29] were used to define the $NNBS_{wt}$ and $NNBS_{Y181C}$.

First-ranking docked conformations for **4d**, **3d** and the experimental binding conformations of TNK-651 are reported in Figure 3.

It can be seen that the two binding modes, in the $NNBS_{wt}$ and $NNBS_{Y181C}$, for **4d** as proposed by AUTODOCK are almost indistinguishable to each other. In either the cases, **4d** binding conformations are characterized by a 'propeller-like' disposition of thymine and 2,6-difluorophenyl rings, with τ_1 and τ_2 values[25,27] of −30.7° and 78.3°.

Experimental conformations of TNK-651 bound into $NNBS_{wt}$/$NNBS_{Y181C}$ differ only for the disposition of the ligand C_5 *iso*-propyl groups, that were found rotated each other of about 180°.[15,29]

A different scenario is observed for the F_2-*S*-DABO **3d**, for which the two RT_{wt} and RT_{Y181C} docked conformations are not exactly superimposable. In fact, while the two molecules are conformationally almost identical, the **3d** RT_{Y181C} bound conformation is slightly rotated in a way that the ligand difluorophenyl moiety seems to approach to Tyr188, thus trying to replace the missing interactions with the mutated Y181C.

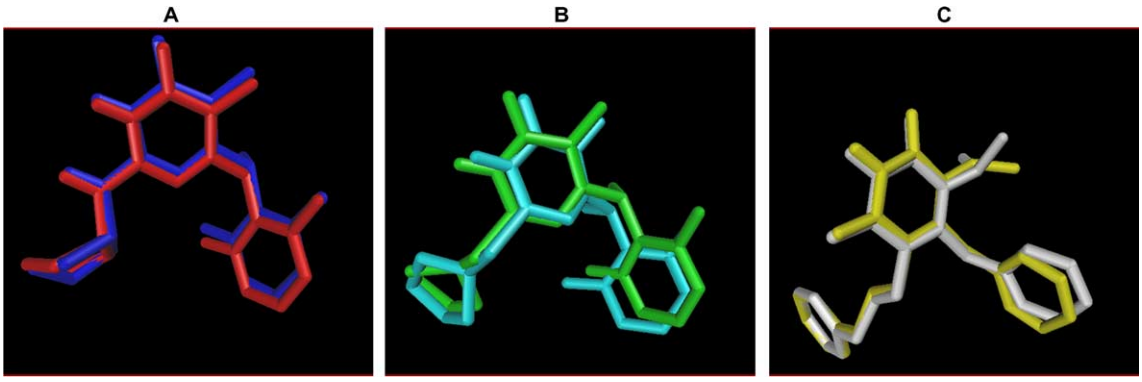


Figure 3. (A): Superimposition of the docked conformations of **4d** in the RT_{wt} (red) and RT_{Y181C} (blue). (B): Superimposition of the docked conformations of **3d** in the RT_{wt} (green) and RT_{Y181C} (cyan). (C): Superimposition of the experimental TNK-651 conformations in the RT_{wt} (white) and RT_{Y181C} (yellow).

The DNA-like hydrogen bonding profile of **4d** bound into $NNBS_{wt}$[28] is still maintained in the RT_{Y181C}/**4d** complex. A deeper inspection of the RT_{Y181C}/**4d** complex reveals a binding mode of the inhibitor in which three potential hydrogen bonds can be observed: (i) a hydrogen bond donated by the 3-NH function of the pyrimidine to the carbonyl oxygen of Lys101 (N–O distance = 3.702 Å), (ii) a hydrogen bond accepted by the C-4 carbonyl oxygen of the pyrimidine from the amidic NH of Lys101 (O–N distance = 2.769 Å), and (iii) a hydrogen bond donated by the C-2 NH group to the carbonyl oxygen of Lys101 (N–O distance = 2.770 Å). This hydrogen bond pattern, compared to that of **3d**, arranges **4d** so as to lift up into the RT pocket (Fig. 4). In such way, the **4d** benzylic methyl group exactly lies in the same spatial position of the C_5 *iso*-propyl of TNK-651, making favorable steric interactions within a hydrophobic pocket formed by the β-methylene of Cys181 ($CH_{3\text{-benz}}$–β-$CH_{2\text{-CYS181}}$ distance = 3.49 Å) and other portion of Val106, Val179, and Tyr188 residues. Finally, the **4d** difluorophenyl moiety is still in good position to make favorable π–π stacking interaction with Tyr188.

At the same time, inspection of the RT_{Y181C}/**3d** complex reveals that the F_2-*S*-DABO inhibitor seats on the Pro236 hairpin, its C_5 methyl group pointing towards the above described hydrophobic pocket. Less important interactions are made between the two benzylic/C_5 methyl groups of **3d** and the Cys181 side chain ($CH_{3\text{-benz}}$–β-$CH_{2\text{-CYS181}}$ distance = 5.45 Å; $CH_{3\text{-C5}}$–β-$CH_{2\text{-CYS181}}$ distance = 4.31 Å), and only appreciable favorable interactions are made by the **3d** difluorophenyl group with Tyr188.

4. Conclusion

2-Alkylamino-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-one derivatives **4**, **5** belonging to the DABO family and bearing different alkyl- and arylamino side chains at the C_2-position of the pyrimidine ring were designed as active against wild type and some relevant mutants of HIV-1. Molecular modeling studies strongly suggested the synthesis of such compounds as they can make an additional hydrogen bond

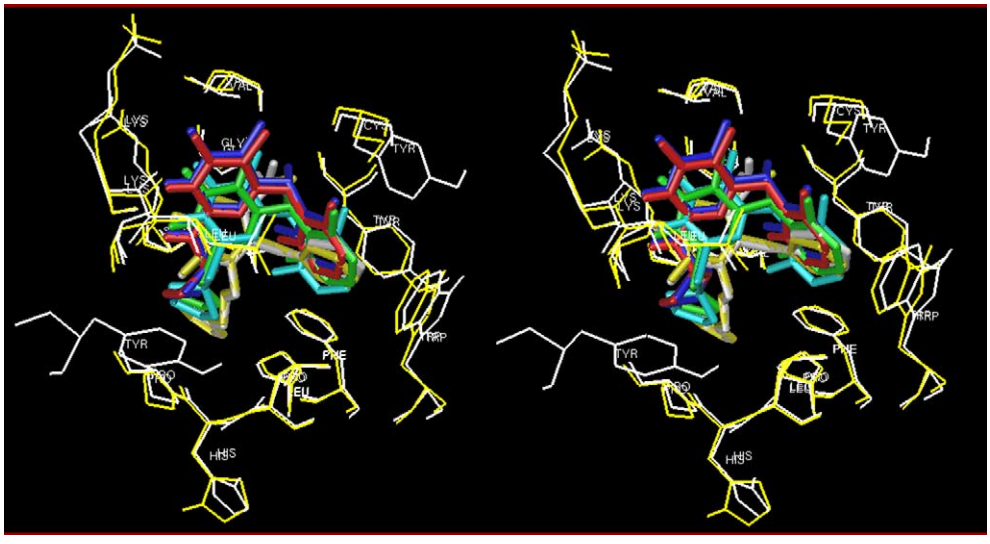


Figure 4. Stereoview of all the six structures in the $NNBS_{wt}$ (white lines) and in $NNBS_{Y181C}$ (yellow lines). The ligands are color coded as in Figure 3.

between the NH group at C_2-position and the carbonyl oxygen of Lys101, with a tighter binding into the enzyme NNBS. Biological evaluation of F_2-*NH*-DABOs indicated the importance of the further anchor point of compounds **4**, **5** into the NNBS: newly synthesized compounds were highly active against both wild type and the Y181C HIV-1 strains. In anti-wt HIV-1 assay, the potency of derivatives did not depend on the size or shape of the C_2-amino side chain, but it associated with the presence of one or two methyl groups, one at C_5-position and the other at the benzylic carbon of the pyrimidine. Differently from that observed with F_2-*S*-DABOs, against wt HIV-1 in the amino-DABO series the double methyl substitution at C_5 and benzylic positions is not necessary for a significant improvement of antiviral activity: thymine and α-methyluracil or α-methylthymine derivatives are almost equally active in reducing wt HIV-1-induced cytopathogenicity in MT-4 cells. Conversely, against the Y181C mutant strain the 2,6-difluorobenzyl-α-methylthymine derivatives **4d**, **5h′**–**n′** were endowed with higher potency and selectivity, in this series both a properly sized C_2–NH side chain and the presence of two methyl groups (at C_5 and benzylic positions) being crucial for high antiviral action. Docking studies performed on wt (RT_{wt}) and Y181C mutant (RT_{Y181C}) form of HIV-1 RT using **3d** and **4d** as F_2-*S*-DABO and F_2-*NH*-DABO representative members, respectively, showed that the further anchor point (hydrogen bond with C_2–NH function) of amino-DABOs, lacking in the thio-analogs, produced a tighter binding of the ligand with the enzyme, so that a mutation at 181 residue (Tyr to Cys) is less effective in reducing the anti-HIV-1 activity (**4d** resistance ratio = 5.3; **3d** resistance ratio = 13).

5. Experimental

5.1. Chemistry

Melting points were determined on a Büchi 530 melting point apparatus and are uncorrected. Infrared (IR) spectra (KBr) were recorded on a Perkin–Elmer 297 instrument. ^{1}H NMR spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). All compounds were routinely checked by TLC and ^{1}H NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values.

The specific examples presented below illustrate general synthetic methods A and B. As a rule, samples prepared for physical (Table 1) and biological studies (Tables 2–5) were dried in high vacuum over P_2O_5 for 20 h at temperatures ranging from 25 to 110 °C, depending on the sample melting point.

5.1.1. General procedure for the preparation of 2-alkylamino-6-[(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-ones 5f–k,o–z,d′–g′,k′–n′. Example: 6-(2,6-difluorophenylmethyl)-3,4-dihydro-2-(2-methoxyethyl)aminopyrimidin-4(3*H*)-one (5h). A mixture of 6-(2,6-difluorophenylmethyl)-3,4-dihydro-2-methylthiopyrimidin-4(3*H*)-one[25] (0.5 g, 1.8 mmol) and 2-methoxyethylamine (5 mL) was heated in a sealed tube at 170 °C for 8 h. After cooling, the crude residue was dissolved in ethyl acetate (50 mL), washed with water (50 mL), 0.5 N HCl (50 mL), and brine (50 mL), and then was evaporated under reduced pressure and purified by column chromatography (silica gel/chloroform). ^{1}H NMR ($CDCl_3$): δ 3.27 (s, 3H, OCH_3), 3.44–3.46 (d, 4H, OCH_2 and NCH_2 overlapped), 3.78 (s, 2H, C_6–CH_2), 5.45 (s, 1H, C_5–H), 6.51 (br s, 1H, C_2–NH exchanged with D_2O), 6.84–6.88 (m, 2H, $C_{3,5}$ Ar–H), 7.13–7.17 (m, 1H, C_4 Ar–H), 11.75 (br s, 1H, CONH exchanged with D_2O). Anal. C, H, N, F.

5.1.2. General procedure for the preparation of 2-alkylamino-6-[(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-ones 5b–e,m,n,b′,c′,i′,j′. Example: 6-(2,6-difluorophenylmethyl)-3,4-dihydro-5-methyl-2-*n*-propylamino pyrimidin-4(3*H*)-one (5m). *n*-Propylammonium-acetate was prepared by slowly adding 2.1 mL (0.036 mol) of glacial acetic acid to 2.9 mL (0.036 mol) of *n*-propyl-amine in an ice bath. 6-(2,6-Difluorophenylmethyl)-3,4-dihydro-5-methyl-2-methylthiopyrimidin-4(3*H*)-one[25] (0.53 g, 1.8 mmol) was added and fused for 5 h at 160 °C. After cooling, the residue was partitioned between water (60 mL) and ethyl acetate (60 mL), and the aqueous phase was extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were washed with brine (3 × 50 mL) and dried. Evaporation of the solvent furnished a crude residue which was purified by column chromatography (silica gel/chloroform). ^{1}H NMR ($CDCl_3$): δ 0.73–0.80 (t, 3H, $NHCH_2$-CH_2CH_3), 1.32–1.43 (m, 2H, $NHCH_2CH_2CH_3$), 2.01 (s, 3H, C_5–CH_3), 3.05–3.08 (m, 2H, $NHCH_2CH_2CH_3$), 3.86 (s, 2H, C_6–CH_2), 6.26 (br s, 1H, $NHCH_2CH_2CH_3$ exchanged with D_2O), 6.80–6.87 (m, 2H, $C_{3,5}$ Ar–H), 7.12–7.20 (m, 1H, C_4 Ar–H), 11.43 (br s, 1H, CONH exchanged with D_2O). Anal. C, H, N, F.

5.1.3. General procedure for the preparation of 2-amino-6-[(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-ones 5a,l,a′,h′,6–9. Example: 6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-2-methylaminopyrimidin-4(3*H*)-one (5a′) and 2-amino-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-3-methylpyrimidin-4(3*H*)-one (6b). Sodium metal (0.58 g, 25.2 mmol) was dissolved in 50 mL of absolute ethanol, and 1-methylguanidine hydrochloride (1.66 g, 15.1 mmol) and ethyl 4-(2,6-difluorophenyl)-3-oxopentanoate[27] (2.56 g, 10.1 mmol) were added to the clear solution. The mixture was heated at reflux for 5 h. After cooling, the solvent was distilled in vacuo at 40–50 °C until dry and the residue was dissolved in a little water (20 mL), made acid with 2 N HCl, and extracted with ethyl acetate (3 × 25 mL). The organic extracts were washed with brine (3 × 50 mL), dried, and evaporated in vacuo, and the residue was purified by chromatography on silica gel eluting with ethyl

acetate–chloroform 3:1. The early eluates deprived of the solvent furnished **6b** as a pure solid; with further elution, pure **5a′** was isolated. Compound **6b**: ^{1}H NMR (DMSO-d_6): δ 1.48–1.51 (d, 3H, ArCHCH_3), 3.21 (s, 3H, NCH_3), 4.06–4.13 (q, 1H, ArCHCH$_3$), 5.51 (s, 2H, C_5–H), 6.99–7.07 (m, 4H, $C_{3,5}$ Ar–H and NH_2), 7.28–7.36 (m, 1H, C_4 Ar–H). Anal. C, H, F, N.

Compound **5a′**: ^{1}H NMR (DMSO-d_6): δ 1.40–1.55 (d, 3H, ArCHCH_3), 2.66–2.68 (d, 3H, NHCH_3), 4.10–4.14 (q, 1H, ArCHCH$_3$), 5.36 (s, 2H, C_5–H), 6.35 (br s, 1H, NHCH$_3$ exchanged with D_2O), 7.03–7.09 (m, 2H, $C_{3,5}$ Ar–H), 7.32–7.36 (m, 1H, C_4 Ar–H), 10.71 (br s, 1H, NHCO exchanged with D_2O). Anal. C, H, F, N.

5.1.4. 6-(2,6-Difluorophenylmethyl)-3,4-dihydro-2-propionylaminopyrimidin-4(3*H*)-one (10). To a cooled (0 °C) solution of **7** (1.26 mmol, 0.3 g) in dry THF (10 mL), triethylamine (1.45 mmol, 0.21 mL) and propionyl chloride (1.39 mmol, 0.13 mL) were added in succession. After stirring for 30 min, the mixture was evaporated under reduced pressure and the residue was partitioned between water (50 mL) and ethyl acetate (50 mL). The organic phase was separated, and the aqueous one was extracted with ethyl acetate (2 × 30 mL). The combined organic solution was washed with brine (2 × 50 mL), dried, and evaporated under reduced pressure. The obtained solid residue was crystallized to furnish pure **10**. ^{1}H NMR ($CDCl_3$): δ 1.15–1.24 (t, 3H, COCH$_2$CH_3), 2.45–2.56 (q, 2H, COCH_2CH$_3$), 3.82 (s, 2H, C_6–CH$_2$), 5.87 (s, 1H, C_5–H), 6.84–6.92 (m, 2H, $C_{3,5}$ Ar–H), 7.15–7.27 (m, 1H, C_4 Ar–H). Anal. C, H, N, F.

5.2. Antiviral assay procedures

5.2.1. Compounds. Compounds were solubilized in DMSO at 200 mM and then diluted into culture medium.

5.2.2. Cells and viruses. MT-4, C8166, H9/IIIB, and CEM cells were grown at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin G and 100 μg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Human immunodeficiency viruses type-1 (HIV-1, III_B strain) and type-2 (HIV-2 ROD strain, kindly provided by Dr. L. Montagnier, Paris) were obtained from supernatants of persistently infected H9/III_B and CEM cells, respectively. HIV-1 and HIV-2 stock solutions had titers of 4.5×10^6 and 1.4×10^5 50% cell culture infectious dose ($CCID_{50}$)/mL, respectively.

5.2.3. HIV titration. Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titer was determined by light microscope scoring of cytopathogenicity after 4 days of incubation and the virus titers were expressed as $CCID_{50}$/mL.

5.2.4. Anti-HIV assays. Activity of the compounds against HIV-1 and HIV-2 multiplication in acutely infected cells was based on the inhibition of virus-induced in MT-4 and C8166 cells, respectively. Briefly, 50 μL of culture medium containing 1×10^4 cells were added to each well of flat-bottom microtiter trays containing 50 μL of culture medium with or without various concentrations of the test compounds. Then 20 μL of an HIV suspension containing 100 (HIV-1) or 1000 (HIV-2) $CCID_{50}$ (50% cell culture infective dose) were added. After a 4 days incubation (5 days for HIV-2) at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[30] Cytotoxicity of the compounds was evaluated in parallel with their antiviral activity. It was based on the viability of mock-infected cells, as monitored by the MTT method.

5.2.5. RT assays. Assays were performed as previously described.[31] Briefly, purified rRT was assayed for its RNA-dependent polymerase-associated activity in a 50 μL volume containing: 50 mM Tris–HCl (pH 7.8), 80 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1 mg mL^{-1} BSA, 0.5 OD_{260} unit mL^{-1} template:primer [poly(rC)–oligo-(dG)$_{12-18}$] and 10 mM [3H]dGTP (1 Ci mmol^{-1}). After incubation for 30 min at 37 °C, the samples were spotted on glass fiber filters (Whatman GF/A), and the acid-insoluble radioactivity was determined.

5.3. Computational studies

All molecular modeling calculations and manipulations were performed using the software packages MACROMODEL 7.1,[32] AUTODOCK 3.0.5,[33] running on Silicon Graphics O2 R10000, IBM compatible Intel Pentium IV 1.4 GHz and AMD Athlon 1.9 GHz workstations. For any minimization the all-atom Amber force field[34] was adopted as implemented in the MACROMODEL package.

The geometry of the RT_{wt} NNBS was taken from the structure of HIV-1 RT/TNK-651 complex filed in the Brookhaven Protein Data Bank[35] (entry code 1rt2). All the residues within 20 Å from any ligand's atom (TNK-651) were used to define the NNBS. Analogously it was done to define the RT_{Y18C} NNBS using the structure of HIV-1 RT_{Y181C}/TNK-651 (PDB entry code 1jla).

The representative compound **4d** was modeled in its *R* configuration as previously reported.[36] Compound **4d** was chosen as F_2-*NH*-DABO representative member for the following reasons: (i) it is one of the most active derivative in both enzymatic and cell based assays; (ii) it has a cyclopentyl moiety attached to the NH–C_2 that reduce the number of conformations, and also no further chiral center is introduced by the cyclopentyl; (iii) it is active against the Y181C mutant strain of HIV.

The starting conformation for docking studies was obtained using molecular dynamics with simulated annealing as implemented in MACROMODEL version 7.1 and

conducted as following: **4d** was energy minimized to a low gradient. The nonbonded cut-off distances were set to 20 Å for both Van der Waals and electrostatic interactions. An initial random velocity to all atoms corresponding to 300 K was applied. Three subsequent molecular dynamic runs were then performed. The first was carried out for 10 ps with a 1.5 fs time step at a constant temperature of 300 K for equilibration purposes. The next molecular dynamic was carried out for 20 ps, during which the system is coupled to a 150 deg thermal bath with a time constant of 5 ps. The time constant represents approximately the half life for equilibration with the bath; consequently the second molecular dynamic command caused the molecule to slowly cool to approximately 150 K. The third and last dynamic cooled the molecule to 50 K over 20 ps. A final energy minimization was then carried out for 250 iterations using conjugate gradient. The minimizations and the molecular dynamics were in all cases performed in aqueous solution. The atom charges automatically assigned by the batchmin module were retained on **4d** for the docking calculations.

For the docking procedure the program AUTODOCK[28,37–45] was used to explore the binding conformation of **4d**. For the docking a grid spacing of 0.375 Å and $60 \times 80 \times 60$ number of points was used. The grid was centered on the mass center of the experimental bound TNK-651 coordinates. The GA-LS method was adopted using the default settings. Amber united atom charges were assigned to the protein using the program ADT (AUTODOCK TOOLS).[46] AUTODOCK generated 100 possible binding conformations for **4d** (see Table 5).

To validate the use of the AUTODOCK program, the docking studies were performed on the reference compound TNK-651 and for comparison purposes also on the previously reported F_2-*S*-DABO **3d**. AUTODOCK successfully reproduced the experimental binding conformations of the reference drug TNK-651 with acceptable root mean square deviation (RMSD) of atom coordinates in either the RT_{wt} (RMSD = 1.01) or RT_{Y181C} (RMSD = 1.08) NNBS.

The results of the AUTODOCK runs that were clustered using an RMSD tolerance of 0.5 Å are summarized in Table 6.

Table 6. Autodock results

Complex	No of distinct conformational clusters	No of multimember conformational clusters	No of conformations in the first ranked cluster
3d/RT_{wt}	5	2	58
3d/RT_{Y181C}	6	4	68
4d/RT_{wt}	17	6	54
4d/RT_{Y181C}	13	8	58
TNK-651/RT_{wt}	75	8	8
TNK-651/RT_{Y181C}	69	8	14

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