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Slow-, Tight-Binding HIV-1 Reverse Transcriptase Non-Nucleoside Inhibitors Highly Active against Drug-Resistant Mutants

Reynel Cancio,[a] Antonello Mai,*[b] Dante Rotili,[b] Marino Artico,[b] Gianluca Sbardella,[c] Imma Clotet-Codina,[d] José A. Esté,[d] Emmanuele Crespan,[a] Samantha Zanoli,[a] Ulrich Hübscher,[e] Silvio Spadari,[a] and Giovanni Maga*[a]

The potent combination therapy in use for the treatment of infection with human immunodeficiency virus type 1 (HIV-1), known as highly active antiretroviral therapy (HAART), has produced sustained reductions in plasma HIV-1 RNA to levels below the limits of detection and has resulted in dramatic reduction in disease progression and mortality in the developed world.[1] However, to maximize the benefits of treatment, suppression of virus replication must be continuous. Failure to completely suppress virus replication, inevitably leads to the selection of drug resistant variants that limits the long term success of antiretroviral therapy.[2]

Actually, three classes of antiretroviral drugs are available: protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTIs), and non-nucleoside reverse transcriptase inhibitors (NNRTIs).[3] Among them, the NNRTIs comprise a very large number of chemically different compounds that bind to a hydrophobic pocket on the HIV-1 RT.[4] These compounds are attractive because of their high potency, low toxicity, and favorable pharmacokinetic properties. Unfortunately, they rapidly select for the outgrowth of HIV-1 strains with mutations that alter the shape of the hydrophobic NNRTI-binding pocket. Resistance to one NNRTI is usually associated with cross-resistance to many other NNRTIs. For this reason the necessity to improve and expand the therapeutic arsenal is still pursued.

Dihydro-alkoxy-benzyl-oxopyrimidines (DABOs) are a potent class of NNRTIs developed in the last decade.[5,6] The structure–activity relationship, supported by molecular modeling, suggested that the C2 substituent is a structural determinant necessary for the antiviral activity of these derivatives. The various DABOs synthesized to date are characterized by different C2-side chains (*S*-DABOs and *NH*-DABOs, Figure 1).[5d–i]

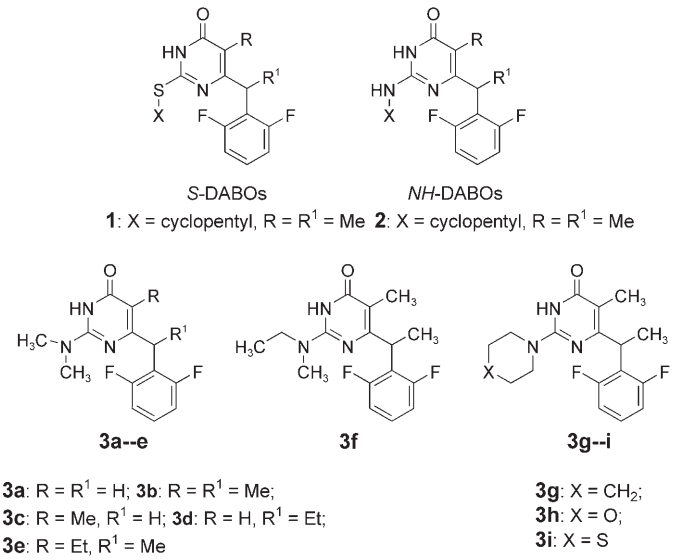


Figure 1. *S*-DABOs, *NH*-DABOs, and *N,N*-DABOs.

To evaluate the resistance pattern of the DABO class of NNRTIs, we decided to study the activity of these pyrimidine derivatives against a panel of HIV-1 mutant strains and against recombinant HIV-1 reverse transcriptases containing single drug-resistant mutations. We focused our attention on the mutations selected from drugs in clinical use (nevirapine, delavirdine, and efavirenz) such as K103N, Y181I, L100I, V106A, V179D, and Y188L.

To establish a correlation between the chemical features of DABOs and the activity against RT-resistant mutants, we evaluated a number of representative compounds of the novel 5-alkyl-2-(*N,N*-disubstituted)amino-6-(2,6-difluorophenylalkyl)-3,4-dihydropyrimidin-4(3*H*)-one series of DABOs (*N,N*-DABOs, **3a–i**) together with the *S*-DABO and *NH*-DABO prototypes **1** and **2**, respectively (Figure 1). Nevirapine (NEV) and efavirenz (EFV) were also tested as references for comparison purpose.

Compounds **3a–i** were prepared by two alternative routes. Condensation of *N,N*-dimethylguanidine hydrogensulfate with the appropriate β-oxoesters in the presence of sodium ethoxide in ethanol afforded the compounds **3a–e** in high yields

[a] *Dr. R. Cancio, Dr. E. Crespan, Dr. S. Zanoli, Dr. S. Spadari, Dr. G. Maga*
Istituto di Genetica Molecolare
Consiglio Nazionale delle Ricerche
via Abbiategrasso 207, 27100 Pavia (Italy)
Fax: (+39) 0382422286
E-mail: maga@igm.cnr.it

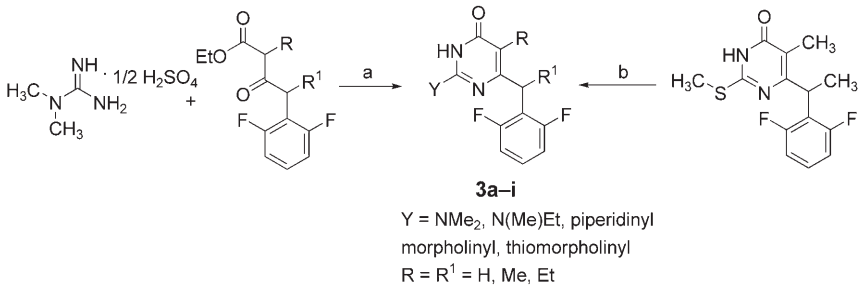
[b] *Prof. A. Mai, Dr. D. Rotili, Prof. M. Artico*
Istituto Pasteur—Fondazione Cenci Bolognetti, Dipartimento di Studi Farmaceutici
Università degli Studi di Roma "La Sapienza"
P. le A. Moro 5, 00185 Roma (Italy)
Fax: (+39) 06491491
E-mail: antonello.mai@uniroma1.it

[c] *Prof. G. Sbardella*
Dipartimento di Scienze Farmaceutiche
Università degli Studi di Salerno
via Ponte Don Melillo, 84084 Fisciano (SA) (Italy)

[d] *Dr. I. Clotet-Codina, Dr. J. A. Esté*
Retrovirology Laboratory IrsiCaixa, Hospital Universitari Germans Trias i Pujol
Universitat Autònoma de Barcelona
08916 Badalona (Spain)

[e] *Dr. U. Hübscher*
Institute of Veterinary Biochemistry and Molecular Biology
University of Zürich-Irchel
Winthertherstrasse 190, 8057 Zürich (Switzerland)

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author. Supporting information includes experimental procedures and characterization data for compounds **3a–i**.



Scheme 1. Reagents and conditions: a) EtONa, EtOH, reflux; b) amine (exc.), sealed tube, 180 °C.

(Scheme 1). Compounds **3 f–i** were obtained by nucleophilic displacement of the C2 methylthio group of the 5-methyl-2-methythio-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydropyrimidin-4(3*H*)-one[5g] with *N*-ethyl-*N*-methylamine, piperidine, morpholine, and thiomorpholine respectively (Scheme 1).

S-DABO (**1**), *NH*-DABO (**2**), and *N,N*-DABO (**3 a–i**) compounds were evaluated against a panel of HIV-1 mutant strains (K103N, Y181C, Y188L, and the clinical isolate IRLL98 containing multiple drug-resistant mutations, Table 1)[7] and against recombinant HIV-1 reverse transcriptases (RTs) either wild type or carrying NNRTI-resistance mutations (K103N, Y181I, L100I, V106A, V179D, and Y188L, Table 3),[8] to characterize their mechanism of action and to evaluate the resistance pattern.

As shown in Table 1, many *N,N*-DABOs were active against wt HIV-1 NL4-3 strain at low nanomolar (**3 c–e** and **3 g–i**) or subnanomolar (**3 b** and **3 f**) concentrations, and retained nanomolar activities against the tested mutant strains (**3 b** and **3 e–h**). In terms of relative resistance (that is —fold of reduced activity), our compounds were similar to nevirapine and efavirenz, however in terms of absolute potencies they proved to be superior. For example, compound **3 f** showed subnanomolar potency against RT wild type (0.3 nM), whereas efavirenz was more than tenfold less potent, scoring 4 nM. Interestingly, compound **3 f** showed an absolute potency of inhibition of 3 nM against the multiresistant strain IRLL98 and 20 nM against the K103N strain. The values for efavirenz were 80 nM and 90 nM, respectively. Also, compound **3 f** showed potencies of inhibition against the two mutants Y181C and Y188L of 2 nM and 30 nM, respectively, whereas efavirenz scored 6 nM and 230 nM, respectively. When the figures for our compounds are compared to the values obtained with nevirapine, the differences are even more pronounced. These data clearly suggest that some of our compounds retained extremely high potencies against several NNRTI-resistant mutants. Thus, irrespective of the loss of potency caused by these mutations with respect to the wild type enzyme, our compounds seem likely to be able to achieve pharmacologically significant potencies when confronted with drug-resistant mutant strains.

To investigate in more detail the mechanism of inhibition of these compounds, we studied the kinetics of inhibitor binding[9] of selected *N,N*-DABOs to HIV-1 RT wild type in comparison with reference compounds such as efavirenz (EFV) and UC781.[10] As reported in Table 2, the association rates (K_{on}) of all the compounds tested were significantly lower than the values for UC781

Table 2. Kinetic constants for binding of *N,N*-DABOs **3 a–i**, efavirenz, and UC781 to HIV-1 RT wild type.[a]

compd	K_i [μM]	K_{on} [$M^{-1}s^{-1}$]	K_{off} [s^{-1}]
3 a	0.078 ± 0.02	$6 \pm 1 \times 10^3$	$6 \pm 1 \times 10^{-4}$
3 b	0.1 ± 0.03	$0.3 \pm 0.02 \times 10^3$	$3 \pm 0.2 \times 10^{-5}$
3 c	0.9 ± 0.1	$9 \pm 1 \times 10^3$	$8 \pm 1 \times 10^{-3}$
3 d	0.1 ± 0.01	$3 \pm 0.1 \times 10^3$	$3 \pm 0.2 \times 10^{-4}$
3 e	0.05 ± 0.01	$4 \pm 0.5 \times 10^3$	$2 \pm 0.1 \times 10^{-4}$
3 f	0.04 ± 0.006	$0.75 \pm 0.09 \times 10^3$	$3 \pm 0.4 \times 10^{-5}$
3 g	0.07 ± 0.01	$0.8 \pm 0.1 \times 10^3$	$6 \pm 1 \times 10^{-5}$
3 h	0.003 ± 0.0005	$2 \pm 0.3 \times 10^3$	$6 \pm 1 \times 10^{-6}$
3 i	0.06 ± 0.01	$4 \pm 0.2 \times 10^3$	$2 \pm 0.3 \times 10^{-4}$
EFV	0.03 ± 0.005	$34 \pm 1 \times 10^3$	$1 \pm 0.4 \times 10^{-3}$
UC781	0.004 ± 0.0005	$2.8 \pm 0.5 \times 10^6$	$9 \pm 1 \times 10^{-3}$

[a] Values are means ±SD determined from at least three independent experiments.

Table 1. Cytotoxicity and anti-HIV-1 activity of *S*-DABO (**1**), *NH*-DABO (**2**), and *N,N*-DABOs (**3 a–i**).[a]

compd	CC_{50} [μM][b]	EC_{50} [μM][c] (fold resistance)[d]				
		NL4-3	IRLL98[e]	K103N	Y181C	Y188L
1	>3	0.0011	0.12 (109)	0.23 (209)	0.10 (91)	>3
2	53	0.002	0.05 (25)	0.19 (95)	0.03 (15)	>10
3 a	>94	0.023	2.0 (87)	>10	3.6 (156)	>10
3 b	>17	0.0003	0.005 (17)	0.05 (167)	0.014 (47)	0.05 (167)
3 c	>89	0.005	0.5 (100)	2.4 (480)	1.82 (364)	>10
3 d	73	0.002	1.6 (800)	0.18 (90)	0.43 (215)	>10
3 e	>81	0.002	0.88 (440)	0.25 (125)	0.10 (50)	0.55 (275)
3 f	81	0.0003	0.003 (10)	0.02 (67)	0.002 (7)	0.03 (100)
3 g	>75	0.007	0.06 (9)	0.52 (74)	0.07 (10)	0.39 (56)
3 h	>74	0.002	0.05 (25)	0.39 (195)	0.09 (45)	0.56 (280)
3 i	>71	0.003	0.06 (20)	1.24 (413)	0.03 (10)	0.86 (287)
NVP	>100	0.08	9.9 (124)	1.8 (23)	0.87 (11)	5.6 (70)
EFV	>0.3	0.004	0.08 (20)	0.09 (22)	0.006 (1.5)	0.23 (57)

[a] Values are means ±SD determined from at least three experiments. [b] Cytotoxic concentration 50, concentration to induce 50% death of noninfected cells, evaluated with the MTT method in MT-4 cells. [c] Effective concentration 50, concentration needed to inhibit 50% HIV-induced cytopathic effect, evaluated with the MTT method in MT-4 cells. [d] Fold-resistance: ratio of EC_{50} value against drug-resistant strain and EC_{50} of the wild type NL4-3 strain. [e] Clinical isolate resistant to NRTIs (lamivudine, emtricitabine) and NNRTIs (NVP, delavirdine, EFV).

Table 3. Inhibitory potencies of DABO derivatives with preincubation in the presence of HIV-1 RT wild type and NNRTI-resistant mutants.[a]

compd	K_i [μM][b]						
	WT_{IIIB}	K103N	Y181I	L100I	V106A	V179D	Y188L
1	0.005	0.03	0.9	0.3	0.5	0.25	1.4
2	0.03	0.03	0.8	1.3	5	3.1	5
3a	0.08	3	3	10	0.5	5	30
3b	0.1	0.5	4	1	ND[c]	2	0.5
3c	0.8	10	>20	10	ND	ND	11
3d	0.1	10	>20	3.3	ND	ND	5
3e	0.05	2	5	4	ND	ND	10
3f	0.04	0.5	10	2.5	ND	ND	4
3g	0.07	0.6	>20	2	ND	ND	3.5
3h	0.003	3.3	>20	10	>20	>20	5.5
3i	0.06	10	>20	5	ND	ND	4
NVP	0.4	7	35	9	10	0.3	18
EFV	0.03	3	0.08	ND	ND	ND	ND

[a] Values are means ±SD determined from at least three experiments. [b] Compound concentration required to inhibit the HIV-1 rRT activity by 50%. [c] ND, not determined.

and EFV. However, the dissociation rates (K_{off}) of all compounds were much slower than for UC781 and EFV, with the exception of **3c**. In particular, the *N,N*-DABOs **3b** and **3f**–**h** displayed 10- to 100-fold slower dissociation rates then the reference compounds. Thus, *N,N*-DABOs behaved as slow-binding tight-binding inhibitors of HIV-1 RT.

As shown in Table 3,[11] all the compounds were differentially affected by NNRTI-resistance mutations. However, in general *N,N*-DABOs showed a better activity profile against the various mutants than nevirapine. The relative -fold resistance values (defined as K_{imut}/K_{iwt}) of the different mutant enzymes are shown in Figure 2 for the representative *N,N*-DABO **3b** in comparison with *S*-DABO (**1**), *NH*-DABO (**2**), and NVP. Compound **3b** scored better than NVP against all mutants, and showed a greatly improved sensitivity profile with respect to the other compounds towards the L100I, Y181I, and Y188L mutant enzymes.

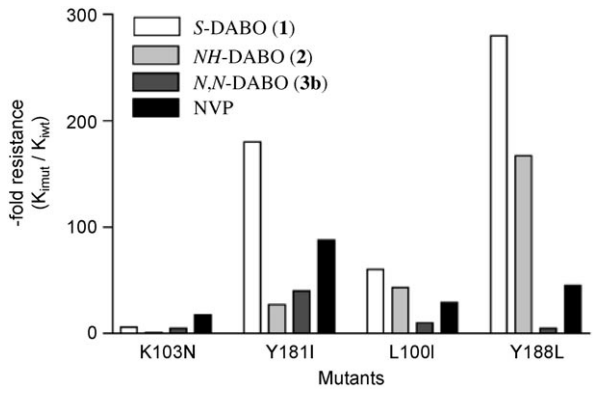


Figure 2. Relative—fold resistance indexes for selected DABO derivatives against HIV-1 RTs carrying NNRTI-resistance mutations. Inhibition potencies (K_i) towards wild type and mutated RTs are reported in Table 3. Relative -fold resistance indexes, defined as K_{imut}/K_{iwt}, for compounds **1**, **2**, **3b**, and nevirapine are shown in the graph.

From molecular modeling studies performed on *S*-DABOs[5f,g] and *NH*-DABOs,[5h,i] a number of contacts of the ligands into the HIV-1 RT non-nucleoside binding site (NNBS) have been reported, suggesting, for example, that tighter binding and increased activity with respect to other NNRTIs was due to a novel hydrogen bond formed with the residue K101. In addition, favorable interactions with residues 106, 179, and 188 were noted. Binding mode analyses on *N,N*-DABOs into the NNBS are still in progress.

Even though the mutations L100I, K103N, Y181I, and Y188L were able to reduce the potency of inhibition of all the *N,N*-DABOs tested, we proved that our molecules were less sensitive to the L100I and Y188L mutations than the *S*- and *NH*-DABOs.

In summary, our results showed that *N,N*-DABOs are characterized by a broad activity spectrum against NNRTI-resistant mutants. Moreover, *N,N*-DABOs display a slow but extremely tight binding to HIV-1 RT, as indicated by the very low K_{off} values of compounds **3b** and **3f**–**h**. Other NNRTIs have been defined as "tight binding", for example the carboxanilide derivative UC781. However, as shown in Table 2, some of our compounds (**3f**–**h**) displayed dissociation rates from 150- to 1500-fold slower than UC781.

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Keywords: anti-HIV-1 agents · dihydro-alkoxy-benzyl-oxopyrimidines (DABOS) · kinetic analysis · non-nucleoside reverse transcriptase inhibitors (NNRTIs) · slow-, tight binding

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[9] Briefly, HIV-1 RT (20–40 nM), was incubated 2 min at 37 °C in a final volume of 4 μL in the presence of 15 nM poly(rA)/oligo(dT) (3′-OH ends), reaction buffer, and 10 mM $MgCl_2$. Inhibitor was then added to a final volume of 5 μL, the concentration at which the fraction of enzyme–inhibitor complex, defined as: [E:I]/[E0] = (1–1/(1+[I]/K_i)), was >90%. Then, 145 μL of a mix containing Buffer A, 10 mM $MgCl_2$, and 10 μM [3H]-dTTP (1 Ci mm^{-1}ol) were added at different time points. After an additional 10 min of incubation at 37 °C, 50 μL aliquots were spotted on GF/C filters and acid-precipitable radioactivity measured as described for the HIV-1 RT RNA-dependent DNA polymerase activity assay.[8] The quantity representing the normalized difference between the amount of dTTP incorporated at the zero time point and at the different time points ((v_0-v_t)/v_0) was then plotted against time. Apparent association rate (K_{app}) values were determined by fitting the experimental data to the single-exponential equation: (v_0-v_t)/v_0 = A(1−$e_{(-K_{appt})}$), where A is a constant and t is time. The true dissociation (K_{off}) and association (K_{on}) rates were then derived from the relationships: K_{app} = K_{on}-(K_i+[I]) and K_i = K_{off}/K_{on}.

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[11] Time-dependent incorporation of radioactive nucleotides into poly(rA)/oligo(dT)$_{10:1}$ at different nucleotide substrate concentrations was monitored by removing 25 μL-aliquots at 2 min time intervals. Initial velocities of the reaction were then plotted against the corresponding substrate concentrations. For inhibition constant (K_i) determination, an interval of inhibitor concentrations between 0.2K_i and 5K_i was used in the inhibition assays. Data were then plotted according to Lineweaver–Burke and Dixon.

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