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BRIEF ARTICLE

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Diarylpyrimidine−Dihydrobenzyloxopyrimidine Hybrids: New, Wide-Spectrum Anti-HIV-1 Agents Active at (Sub)-Nanomolar Level

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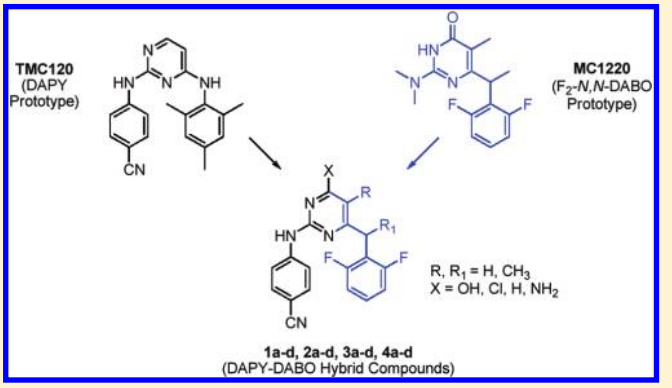
Supporting Information

ABSTRACT: Here, we describe a novel small series of non-nucleoside reverse transcriptase inhibitors (NNRTIs) that combine peculiar structural features of diarylpyrimidines (DAPYs) and dihydro-alkoxy-benzyl-oxopyrimidines (DABOs). These DAPY−DABO hybrids (**1**−**4**) showed a characteristic SAR profile and a nanomolar anti-HIV-1 activity at both enzymatic and cellular level. In particular, the two compounds **4d** and **2d**, with a (sub)nanomolar activity against wild-type and clinically relevant HIV-1 mutant strains, were selected as lead compounds for next optimization studies.



INTRODUCTION

The worldwide spread of AIDS (acquired immunodeficiency syndrome), an epidemic disease in continuous development, has required and still requires potent antiretroviral chemotherapeutic agents for reducing the number of deaths caused by HIV-1 (human immunodeficiency virus type 1), the etiological agent of AIDS. Global estimates of WHO/UNAIDS showed 33.4 million people infected with HIV/AIDS at the end of 2008, with 2.7 million newly infected and 2.0 million deaths.[1]

The current therapy against AIDS is based on seven classes of anti-HIV drugs: the nucleoside and nucleotide reverse transcriptase inhibitors (indicated as NRTIs and NtRTIs, respectively),[2,3] the non-nucleoside reverse transcriptase inhibitors (NNRTIs),[4] the protease inhibitors (PIs),[5] the integrase inhibitor (INI) raltegravir,[6] the chemokine (C−C motif) receptor 5 (CCR5) inhibitor maraviroc,[7,8] and the fusion inhibitor (FI) enfuvirtide.[7,8] NRTIs, NtRTIs, NNRTIs, and PIs are actually mixed in the highly active antiretroviral therapy (HAART), which dramatically reduces the incidence of AIDS infection and death. Despite the fact that HAART combination regimens have significantly decreased the morbidity and mortality among patients with HIV infections, slowing the viral replication to very low levels, they are still unable to eradicate the virus.[9] So, the needed long-term or permanent use of anti-AIDS drugs induces the selection of drug-resistant viral mutants and the emergence of undesired metabolic side effects. Moreover and unfortunately, when individuals develop resistance to one antiretroviral agent within a class there is often, but not always, development of cross-resistance to other agents of the same class.

Despite their chemical diversity, NNRTIs bind to a common allosteric site of HIV-1 reverse transcriptase (RT) and noncompetitively inhibit DNA polymerization. NNRTIs currently used in HAART include nevirapine, delavirdine, efavirenz, and etravirine (TMC125), which has been approved by FDA on January 2008.[10,11]

Since 1992, our research group has been working to develop effective anti-HIV drugs and our efforts have resulted in the discovery of the dihydro-alkyloxy-benzyl-oxopyrimidine (DABO) family of NNRTIs.[12] Since the first DABO derivatives, many structural modifications have been introduced in order to obtain more potent and selective compounds, which led to the discovery of some series of excellent DABO compounds such as F_2-*S*-DABOs[13−16] and F_2-*N*,*N*-DABOs[17−21] (Chart 1) that are

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Chart 1. Chemical Structures of Selected DAPY and DABO Derivatives

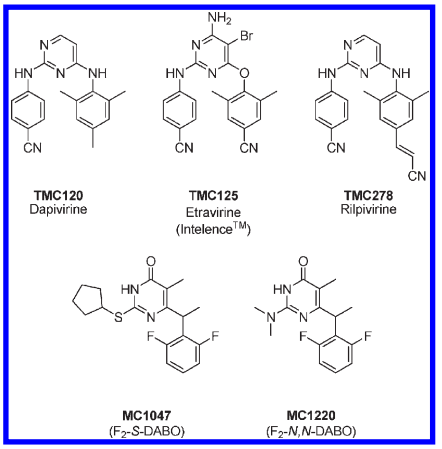
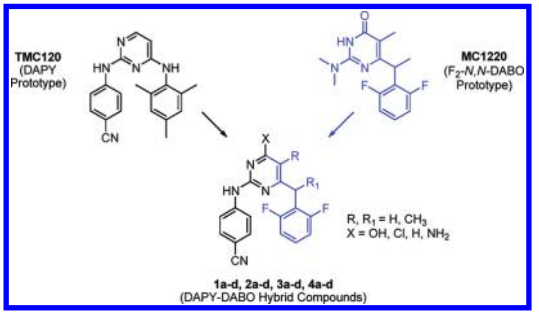


Chart 2. Design of DAPY−DABO Hybrid Compounds

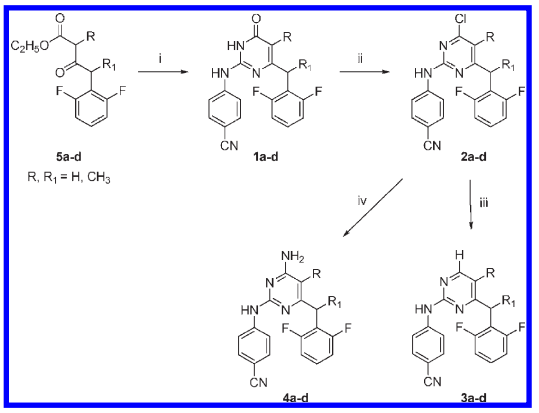


endowed with inhibitory potencies in the low nanomolar or subnanomolar range without a significant cytotoxicity at high concentrations.

Diarylpyrimidine (DAPY) derivatives are one of the most successful family of NNRTIs developed so far, as confirmed by the recent approval of etravirine for the clinical use.[10] The innate flexibility between their characteristic two aromatic rings allows these compounds to adopt multiple conformations within the RT non-nucleosidic binding site, explaining their potent activity against many clinically relevant resistant viral mutant strains. SAR studies on the DAPY series had also found that the *para*-cyanoaniline substitution at the C-2 pyrimidine ring position is crucial for a significant inhibitory activity (Chart 1).[22−25]

To obtain NNRTIs highly active against wild type and mutant HIV-1 strains, we prepared four brief series of compounds (**1**−**4**) characterized by the *para*-cyanoaniline group typical of DAPYs at the C-2 of the pyrimidine ring and the 2,6-difluorobenzyl group peculiar of DABOs at the C-6 position. These series differed each other for the groups at the C-4 pyrimidine ring position which were characteristic either of DABOs (−OH, **1a**−**d**) or of DAPYs (−Cl, **2a**−**d**; −H, **3a**−**d**; $-NH_2$, **4a**−**d**). We also studied the effects of the methyl substitution at the C-5 pyrimidine position and at the methylene group of the benzylic moiety in C-6 of these arylamino benzyl pyrimidines that can be considered a sort of DAPY-DABO hybrid compounds (Chart 2).

Scheme 1[a]



[a] (i) EtONa, dry EtOH, reflux; (ii) $POCl_3$/DMF, dry DMF, rt; (iii) zinc dust, 33% NH_4OH, THF-dioxane, reflux; (iv) 7 N methanolic ammonia, dry THF, 120 °C, Parr high pressure reactor.

CHEMISTRY

Condensation of the β-oxoesters **5a**−**d**, prepared as reported previously,[14] with the 1-(4-cyanophenyl)guanidine nitrate[26] in the presence of sodium ethoxide in dry ethanol under reflux conditions, afforded the 2-(4-cyanoanilino)-pyrimidin-4(3*H*)-ones **1a**−**d**. Further Vilsmeier reaction with phosphorus oxychloride and *N*,*N*-dimethylformamide at room temperature in dry *N*,*N*-dimethylformamide performed on **1a**−**d** yielded their 4-choropyrimidine analogues **2a**−**d**. The 4-unsubstituted-(**3a**−**d**) and the 4-aminopyrimidine (**4a**−**d**) derivatives were synthesized from the appropriate **2a**−**d** by reductive dehalogenation with zinc dust in a mixture of THF−dioxane in the presence of ammonium hydroxide under reflux conditions and by nucleophilic substitution with methanolic ammonia in a Parr high pressure reactor, respectively (Scheme 1).

RESULTS AND DISCUSSION

The DAPY−DABO hybrid compounds **1**−**4** were tested in MT-4 cells to evaluate their cytotoxicity and their capability to inhibit the HIV-induced cytopathic effect (HIV-1 strain: NL4-3) by 50%. The compounds were also tested against a panel of clinically relevant HIV-1 mutant strains (K103N, Y181C, and Y188L). Nevirapine (NVP), efavirenz (EFV), dapivirine (TMC120), and MC1220 (the F_2-*N*,*N*-DABO prototype) were also tested as reference drugs (Table 1).

The highest inhibitory activity (EC_{50}, concentration needed to inhibit the HIV-induced cytopathic effect by 50%) against wild type HIV-1 was displayed by the compounds with a free amino group ($-NH_2$) at the C-4 position of the pyrimidine ring (**4a**−**d**, values ranging from 0.2 to 0.6 nM), apart from their methyl substitution at the C-5 and/or C-6 benzylic positions. All of them were more than 10-fold more potent than efavirenz, and showed against wild type HIV-1 a potency greater than or comparable to TMC120 and MC1220, the DAPY and DABO prototypes, respectively. In addition, **4a**−**d** showed CC_{50} (compound concentration toxic for 50% cells) values >10- and 2.5-fold higher than TMC120 and MC1220, respectively, exhibiting in such a way up to 45-fold higher selectivity indexes (SI, CC_{50}/EC_{50}). Similar subnanomolar WT HIV-1 inhibiting activity was displayed by the pyrimidine C-4 unsubstituted **3a**,**c**,**d** (**3b** was about 6 times

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Table 1. Cytotoxicity and Anti-HIV-1 Activity of 1−4 against WT (NL4-3) and Clinically Relevant HIV-1 Mutant Strains[a]

				EC_{50},[b] nM (fold resistance)[c]					
compd	X	R	R_1	NL4-3	K103N	Y181C	Y188L	CC_{50},[d] nM	SI[e]
1a	OH	H	H	344	290 (*0.8*)	344 (*1*)	>2 500	>2 500	>7.3
1b	OH	Me	H	244.1	1 391 (*6*)	>2 838	>2 838	>2 838	>11.6
1c	OH	H	Me	9.1	56.8 (*6*)	88 (*10*)	652.8 (*72*)	>2 838	>311.9
1d	OH	Me	Me	2.9	61.14 (*21*)	45.58 (*16*)	230.4 (*79*)	1 064	366.9
2a	Cl	H	H	13.4	171 (*13*)	561 (*42*)	1 401 (*105*)	>2 803	>209.2
2b	Cl	Me	H	21.6	739 (*23*)	2 535 (*80*)	>2 697	>2 697	>124.9
2c	Cl	H	Me	1.5	51.24 (*34*)	153.7 (*102*)	836.1 (*557*)	>2 697	>1 798
2d	Cl	Me	Me	2.3	2.3 (*1*)	44 (*22*)	60 (*30*)	>13 000	>5 652.2
3a	H	H	H	0.5	68.2 (*136*)	60.2 (*120*)	224.9 (*450*)	>77 564	>155 128
3b	H	Me	H	3.3	88.3 (*27*)	357.7 (*108*)	1 016.8 (*308*)	>74 330	>22 524.3
3c	H	H	Me	0.5	27.3 (*55*)	44.3 (*89*)	100.2 (*200*)	37 492	74 984
3d	H	Me	Me	0.6	51.9 (*86*)	42.5 (*71*)	31.4 (*52*)	71 354	118 923.3
4a	NH_2	H	H	0.3	14.2 (*47*)	71.4 (*238*)	108.2 (*361*)	44 585.9	148 619.7
4b	NH_2	Me	H	0.2	8.2 (*41*)	70.3 (*352*)	151.1 (*756*)	45 936.7	229 683.5
4c	NH_2	H	Me	0.3	8.2 (*27*)	44.7 (*149*)	66.6 (*222*)	46 079	153 596.7
4d	NH_2	Me	Me	0.6	34.7 (*58*)	39.9 (*66*)	27.4 (*46*)	40 505.9	67 509.8
TMC120				0.6	1.2 (*2*)	1.2 (*2*)	333.9 (*556*)	3 035.8	5 059.7
MC1220				0.3	50 (*167*)	14 (*47*)	50 (*167*)	>17 000	>56 667
NVP				131.4	6 121 (*47*)	>7 510	>7 510	7 510	57.15
EFV				7.3	343.4 (*327*)	10.1 (*1.4*)	1 615 (*221*)	3 167.8	433.9

[a] Values are means determined from at least two experiments. [b] Effective concentration 50, concentration needed to inhibit 50% HIV-induced strain. [c] Fold change of the corresponding EC_{50} and the EC_{50} value of the WT NL4-3 strain. [d] Cytotoxic concentration 50, concentration to induce 50% death of noninfected cells, evaluated with the MTT method in MT-4 cells. [e] Selectivity index, CC_{50}/EC_{50}(NL4-3).

less potent), also in these cases joined to very low cytotoxicity and high SI. The C-4 chloro- and hydroxy-substituted compounds were still active in the single digit nanomolar range against the wild type HIV-1 when substituted with a methyl group in the C-6 benzylic position (**2c**,**d** and **1c**,**d**), while showing a decrease of potency in its absence.

The C-4 chlorosubstituted **2d** showed very promising single digit nanomolar inhibiting activity against the K103N mutant strain [EC_{50}^{K103N} (nM)/fold resistance: 2.3/*1*], which was >170- and 22-fold higher than efavirenz and MC1220, respectively, and comparable to TMC120. For this activity, it seems important the presence of two methyl groups both at the C-5 and at the C-6 benzylic positions of the pyrimidine ring. In fact, the removal of one or both of these groups is responsible of a decrease of inhibition against this mutant. Also, the C-4 aminosubstituted **4a**−**d** showed K103N mutant inhibiting activity in the low nanomolar range (with the exception of **4d**), while the unsubstituted (**3a**−**d**) and the hydroxysubstituted (**1a**−**d**) derivatives inhibited the K103N mutant at high nanomolar or low micromolar (**1b**) level.

Among the tested derivatives, the C-4 unsubstituted **3d** and the C-4 amino analogue **4d**, containing two methyl groups at both the C-5 and C-6 benzylic positions of the pyrimidine ring, were the most efficient agents against the Y181C and Y188L HIV-1 mutants. Indeed, they displayed nanomolar inhibitory potencies against the two HIV-1 variants, and when compared to TMC120 and MC1220, they were less potent against the Y181C and more effective against the Y188L mutant strain. The order of potency of the four different series (**1**−**4**) against the Y181C and Y188L mutants resembled the activity against the wild type HIV-1: C4−NH_2 > −H > −Cl > −OH.

Table 2. Cytotoxicity and Anti-HIV-1 Activity of Selected Compounds 2d and 4d against WT HIV-1 (NL4-3) and Multidrug Resistant (MDR) HIV-1 Strain IRLL98 Bearing K101Q, Y181C, and G190A Mutations[a]

	EC_{50},[b] nM (fold resistance)[c]		
compd	NL4-3	IRLL98	CC_{50},[d] nM
2d	2.6	7.8 (*3*)	>13 000
4d	0.08	0.82 (*10*)	>13 700
TMC120	0.03	0.3 (*10*)	1 821.5
MC1220	3.41	204.6 (*60*)	>17 000
NVP	226.1	>18 850 (>*83*)	>18 850
EFV	3.17	633.5 (*200*)	3 167.8

[a] Values are means determined from at least two experiments. [b] Effective concentration 50, concentration needed to inhibit 50% HIV-induced strains. [c] Fold change of the corresponding EC_{50} and the EC_{50} value of the WT NL4-3 strain. [d] Cytotoxic concentration 50, concentration to induce 50% death of noninfected cells, evaluated with the MTT method in MT-4 cells.

Selected 4-chloro- and 4-amino hybrids **2d** and **4d** were then tested against the HIV-1 IRLL98 strain, bearing the K101Q, Y181C, and G190A mutations and being resistant to NVP, EFV, and delavirdine. TMC120, MC1220, NVP, and EFV were also tested as reference drugs (Table 2). In this assay, **2d** displayed nanomolar activity against both WT and IRLL98 strains, with only 3-fold lower activity against the triple, multidrug resistant mutant, while **4d** was potent at subnanomolar level against the two tested strains, showing similar potency and fold-resistance as TMC120. Both **2d** and **4d**

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Table 3. Inhibitory Activity of 1−4 against HIV-1 RT Wild Type and NNRTI-Resistant Mutants[a]

				ID_{50},[b] nM (fold resistance)[c]				
compd	X	R	R_1	WT	K103N	Y181I	Y188L	L100I
1a	OH	H	H	0.8	2 600 (*3 250*)	56 000 (*70 000*)	1 238 (*1 547*)	1 600 (*2 000*)
1b	OH	Me	H	200	21 440 (*107*)	>80 000	>80 000	ND[d]
1c	OH	H	Me	28	347 (*12*)	12 260 (*438*)	4 171 (*149*)	ND
1d	OH	Me	Me	0.4	125 (*312*)	1 400 (*3 500*)	69 (*172*)	1 000 (*2 500*)
2a	Cl	H	H	59	4 365 (*74*)	>80 000	>80 000	ND
2b	Cl	Me	H	17	445 (*26*)	>40 000	6 300 (*371*)	ND
2c	Cl	H	Me	12	286 (*24*)	28 550 (*2 379*)	2 847 (*237*)	ND
2d	Cl	Me	Me	8.8	71 (*8.1*)	3 400 (*386*)	23 (*2.6*)	150 (*17*)
3a	H	H	H	6	500 (*83*)	7 474 (*1 246*)	1 836 (*306*)	671 (*112*)
3b	H	Me	H	9	262 (*29*)	>80 000	>80 000	2 957 (*329*)
3c	H	H	Me	9	248 (*28*)	5 906 (*656*)	766 (*85*)	664 (*74*)
3d	H	Me	Me	6.9	154 (*22*)	5 000 (*725*)	294 (*43*)	128 (*19*)
4a	NH_2	H	H	11	169 (*15*)	17 260 (*1 569*)	3 079 (*280*)	1 509 (*137*)
4b	NH_2	Me	H	11	90 (*8*)	22 070 (*2 006*)	3 462 (*315*)	603 (*55*)
4c	NH_2	H	Me	11	109 (*10*)	11 080 (*1 007*)	1 623 (*147*)	765 (*69*)
4d	NH_2	Me	Me	14	22 (*1.6*)	1 683 (*120*)	146 (*10*)	282 (*20*)
TMC120				7	100 (*14*)	>1 000 (*>143*)	380 (*54*)	90 (*13*)
MC1220				100	500 (*5*)	4 000 (*40*)	500 (*5*)	1 000 (*10*)
NVP				400	7 000 (*17*)	35 000 (*87*)	18 000 (*45*)	9 000 (*17*)
EFV				30	3 000 (*100*)	80 (*3*)	ND	ND

[a] Values are means determined from at least three experiments. [b] Inhibitory dose 50, compound dose required to inhibit the HIV-1 rRT. [c] Fold change of the corresponding EC_{50} and the EC_{50} value of the WT HIV-1 NL4-3 strain. [d] ND, not determined.

were much more efficient than MC1220 and than, as expected, NVP and EFV in inhibiting the HIV-1 IRLL98 strain.

The DAPY−DABO hybrid derivatives **1**−**4** were also tested against recombinant WT HIV-1 RT as well as against a panel of recombinant RTs carrying known NNRTI-resistance mutations (K103N, Y181I, Y188L, and L100I) (Table 3).

In this assay, the majority of the tested derivatives (**1a**, **1d**, **2b**−**d**, **3a**−**d**, and **4a**−**d**) showed ID_{50} (inhibitory dose 50, compound dose required to inhibit the HIV-1 rRT activity by 50%) values at low nanomolar level against WT RT, in some cases also reaching subnanomolar concentration (**1a** and **1d**). As a rule, the substitution pattern of the hybrids at the C-4 (−OH, −Cl, −H, or $-NH_2$), C-5 (−H or $-CH_3$), and C-6 benzylic (−H or $-CH_3$) positions of the pyrimidine ring played only modulatory effects on the WT RT inhibiting activity of the derivatives. Nevertheless, in all the **1**−**4** series, the double methyl substitution at both C-5/C-6 benzylic position was crucial in terms of potency against the mutated HIV-1 RTs. Indeed, compounds **1d**, **2d**, **3d**, and **4d** displayed the highest inhibitory activity (within the corresponding series) against the K103N, Y181I, Y188L, and L100I mutated RTs, with a low fold resistance respect to WT RT. In particular, the C-4 chloro- (**2d**) and the C-4 amino- (**4d**) derivatives were more efficient than TMC120 against the K103N RT, while the C-4 hydroxy- (**1d**) and the C-4 unsubstituted (**3d**) compounds were less effective. All the four pyrimidines (**1d**, **2d**, **3d**, and **4d**) displayed higher activity than TMC120 against the Y181I RT, while they were less potent against the L100I RT. Finally, **1d**, **2d**, **3d**, and **4d** inhibited all the mutated RTs up to 23-fold tighter than MC1220, with **2d** and **4d** being again the most effective in the comparison (for example, they were 22 (**2d**) or 23 (**4d**) times more potent than MC1220 in inhibiting the Y188L or the K103N RT, respectively).

In conclusion, in the present paper, we report a novel series of DAPY−DABO hybrids (**1**−**4**) endowed with high, wide-spectrum anti-HIV-1 activity both in cellular and enzyme assays. The new compounds have been obtained by combination of peculiar chemical features of the two well-known families of pyrimidine-containing NNRTIs (DAPYs and DABOs) and showed a distinct, characteristic structure−activity relationship (SAR) profile. Among these highly potent compounds, all the derivatives carrying the double pyrimidine C-5/C-6 benzylic position methyl substitution (**1d**, **2d**, **3d**, and **4d**) displayed the highest inhibitory activity both in cell and enzyme assays, exhibiting an inhibition profile in general better than that of TMC120 and MC1220, the two DAPY and DABO prototypes. Among them, the pyrimidine C-4 choro- (**2d**) and C-4 amino (**4d**) derivatives showing efficient inhibition at (sub)nanomolar level against the tested WT/mutant HIV-1 strains and the WT/mutated HIV-1 RTs were selected as lead compounds for next optimization studies.

EXPERIMENTAL SECTION

Anti-HIV Activity in Lymphoid Cells. Biological activity of the compounds was tested in the lymphoid MT-4 cell line (received from the NIH AIDS Reagent Program) against the wt HIV-1 NL4−3 strain and three different HIV-1 strains, as described before.[27−29] Briefly, MT-4 cells were infected with the appropriate HIV-1 strain (or mock-infected to determine cytotoxicity) in the presence of different drug concentrations. At day five postinfection, a tetrazolium-based colorimetric method (MTT method) was used to evaluate the number of viable cells. The HIV-1 K103N, Y181C, or Y188L mutant were received from the Medical Research Council Centralised Facility for AIDS Reagents, Herfordshire, UK.

Anti-HIV Reverse Transcriptase Assays. RNA-dependent DNA polymerase activity was assayed as described[30] in the presence of 0.5 μg of poly(rA)/oligo(dT)$_{10:1}$ (0.3 μM 3′-OH ends), 10 μM [3H]-dTTP (1 Ci/mmol) and 2−4 nM RT in the presence of 8% final concentration of DMSO.

Reagents. [^{3}H]-dTTP (40 Ci/mmol) was from Amersham and unlabeled dNTP's from Boehringer. Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka. The homopolymer poly(rA) (Pharmacia) was mixed at weight ratios in nucleotides of 10:1, to the oligomer oligo-(dT)$_{12-18}$ (Pharmacia) in 20 mM Tris-HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA, heated at 65 °C for 5 min and then slowly cooled at room temperature.

Proteins. Recombinant proteins expression and purification was as described.[30] All enzymes were purified to >95% purity.

RT Inhibition Assays. 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 (ID_{50}) determination, an interval of inhibitor concentrations between 0.2 ID_{50} and 5 ID_{50} was used in the inhibition assays. ID_{50} values were determined with computer-aided curve fitting of the experimental data to a fully noncompetitive model. Curve fitting was performed with the program GraphPad Prism 3.0.

■ ASSOCIATED CONTENT

Supporting Information. Chemical and physical data of compounds **1**−**4**. Experimental chemical procedures. Elemental analyses of compounds **1**−**4**. ^{1}H NMR data for compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AIDS, acquired immunodeficiency syndrome; DABOs, dihydro-alkoxy-benzyl-oxopyrimidines; DAPYs, diarylpyrimidines; EFV, , efavirenz; F$_2$-*N*,*N*-DABOs, 5-alkyl-2-(*N*,*N*-disubstituted)amino-6-(2,6-difluorophenylalkyl)pyrimidin-4(3*H*)ones; HIV, human immunodeficiency virus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *NH*-DABOs, dihydro-alkyl-amino-benzyl-oxopyrimidines; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NVP, nevirapine; RT, reverse transcriptase; SAR, structure−activity relationship; *S*-DABOs, dihydro-alkylthio-benzyl-oxopyrimidines; WT, wild type

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