6-[1-(2,6-Difluorophenyl)ethyl]pyrimidinones Antagonize Cell Proliferation and Induce Cell Differentiation by Inhibiting (a Nontelomeric) Endogenous Reverse Transcriptase

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Received July 28, 2005

Abstract: Two 2,6-difluoro-DABO derivatives (MC 1047, **1**, and MC 1220, **2**, respectively) were tested against endogenous, nontelomeric reverse transcriptase (endo-RT) in human differentiating cell systems to investigate their antiproliferative and cytodifferentiating activity. The two compounds significantly reduced cell proliferation and facilitated the morphological differentiation of cells. These results propose F_2-DABOs as useful tools in preventive and/or curative therapy to counteract the loss of differentiation in dedifferentiating pathologies and as antiproliferative drugs in tumor therapy.

The recent realization that retrotransposons can reshape the genome and contribute to modulation of gene expression[1,2] led to reconsideration of the importance of these elements. As a matter of fact, human DNA is significantly (45%) composed of retrotransposable elements, all (but the Alu family) endowed with a reverse transcriptase (RT)-coding gene.[2,3] Growing evidence indicates that RT-coding genes are expressed at low levels, if at all, in differentiated nonpathological tissues; in contrast, high expression is distinctive of germ cells, embryos, embryonic tissues, and undifferentiated and transformed cells, suggesting that levels of RT expression are linked to the proliferative potential of the cell.[2] Unscheduled activity of retrotransposons and endogenous retroviruses (ERVs) is implicated in a variety of diseases, including cancer, while inactivation of specific RT-encoding elements using antisense oligonucleotides or ribozymes inhibited proliferation of human and murine cell lines.[2]

Nevirapine and efavirenz, two well-known nonnucleoside reverse transcriptase inhibitors (NNRTIs), are able to modulate cell growth and differentiation in several cell lines.[2,4−6] They reduce proliferation, induce morphological differentiation, and reprogram gene expression.[2] These features are reversible upon discontinuation of the anti-RT treatment, suggesting that RT contributes to an epigenetic level of control. Most importantly, inhibition of RT activity in vivo antagonizes tumor growth in animal experiments. Moreover, pretreatment with these RT inhibitors attenuated the tumorigenic phenotype of prostate carcinoma cells inoculated in nude mice.[2] On the basis of these data, the endogenous RT can be regarded as an epigenetic regulator of cell differentiation and proliferation and may represent a novel target in cancer therapy.[2]

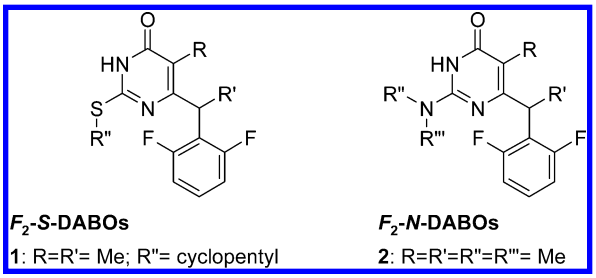
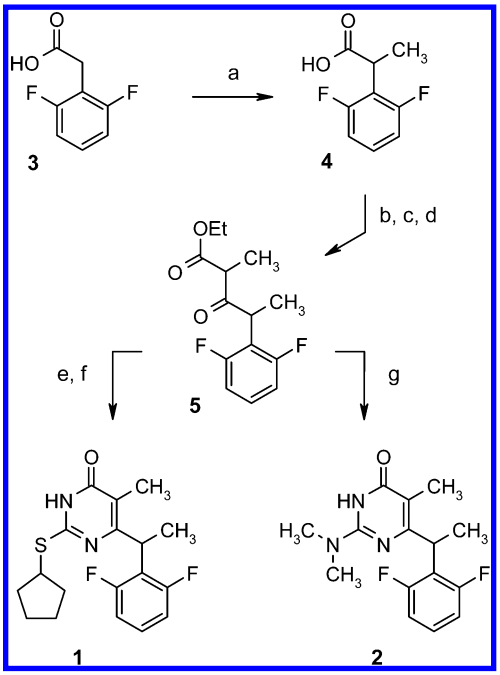


Figure 1. F_2-DABOs.

Scheme 1[a]



[a] Reagents and conditions: (a) *n*-BuLi, MeI, THF, −10 °C; (b) $SOCl_2$, reflux; (c) potassium ethyl 2-methylmalonate, $MgCl_2$, TEA, CH_3CN; (d) HCl 12%; (e) thiourea, Na, EtOH, reflux; (f) *c*-PentBr, K_2CO_3, DMF; (g) *N,N*-dimethylguanidine sulfate, Na, EtOH, reflux.

2,6-Difluoro-S-DABOs and -N-DABOs (F_2-*S*-DABOs and F_2-N-DABOs, Figure 1) are the latest generations of a class of NNRTIs developed by our group after a decade of rationale lead optimization studies.[7−17] In both cell-based and enzymatic assays they are active at low nanomolar concentrations against HIV-1 RT, thus being more potent than nevirapine and comparable to efavirenz. With the aim to investigate the antiproliferative and cytodifferentiating activity of other NNRTIs, we chose[18] MC 1047 (**1**) and MC 1220 (**2**), respectively, as representatives of the F_2-S-DABO and F_2-N-DABO classes and tested them against endo-RT.

Derivatives **1**[13] and **2** (Scheme 1) were prepared, both as racemic mixtures,[19] starting from the same intermediate: in fact, ethyl 4-(2,6-difluoro)phenyl-2-methyl-3-oxopentanoate (**5**) was condensed, respectively, with

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10.1021/jm0507330 CCC: $30.25
Published on Web 09/30/2005

Table 1. Inhibition of Proliferation by F_2-DABOs[a]

compound (μM)	alive cells (%)[b]
CTRL	100
1 (10 μM)	77
1 (25 μM)	72
1 (40 μM)	65
1 (50 μM)	14
2 (10 μM)	91
2 (25 μM)	86
2 (40 μM)	42
2 (50 μM)	30
NEV (350 μM)	35
EFV (15 μM)[c]	51

[a] Cell growth in human A-375 melanoma cultures treated with DMSO (control), **1**, **2**, nevirapine (NEV), and efavirenz (EFV). [b] Cells were harvested and counted after 96 h. Counted cells are expressed as the % of controls, taken as 100. Values represent pooled data from three experiments. [c] Maximum testable concentration.

Table 2. Effect of F_2-DABOs on Cell Death[a]

compound (μM)	alive cells (%)[b]	necrotic cells (%)[b]	apoptotic cells (%)[b]
control	97.6	0.2	2.2
1 (10 μM)	96.7	1	2.3
1 (25 μM)	96.4	1	2.6
1 (40 μM)	98.7	0.3	1
1 (50 μM)	80	7.2	12.8
2 (10 μM)	97.4	0.8	1.8
2 (25 μM)	96.3	1	2.3
2 (40 μM)	96	0.9	3.1
2 (50 μM)	89	2.6	8.4

[a] Cell death in human A-375 melanoma cultures treated with DMSO (control), **1**, and **2** for 96 h. [b] Counted cells are expressed as the % of controls, taken as 100. Values represent pooled data from three experiments.

thiourea in the presence of sodium ethoxide to yield, after S-alkylation in dry DMF with bromocyclopentane in the presence of potassium carbonate, compound **1** or with *N,N*-dimethylguanidine sulfate in the same alkaline medium to afford compound **2**. Preparation of the above-mentioned β-oxoester **5** was accomplished starting from the methylation of (2,6-difluoro)phenylacetic acid **3** with methyl iodide in the presence of *n*-butyllithium in anhydrous THF to afford 2-(2,6-difluoro)-phenylpropionic acid **4**. The latter acid was treated with thionyl chloride and then reacted with potassium monoethyl-2-methylmalonate in the presence of the magnesium dichloride/triethylamine system[13] to yield, after decarboxylation with hydrochloric acid 12%, the required intermediate **5**.

Compounds **1** and **2** (DMSO solutions) were tested in human A-375 melanoma (ATCC-CRL-1619) cell lines in comparison with nevirapine and efavirenz as reference drugs.

As shown in Table 1, both of them resulted more effective than references in reducing cell proliferation. It is particularly noteworthy that test compounds were more active than nevirapine at concentrations 7-fold lower[20] (compare inhibition at 50 μM of tested compounds with inhibition at 350 μM of reference drug).

We next asked whether this antiproliferative effect of the two F_2-DABO could be due to A-375 cell death. Combined staining with propidium iodide (PI) to reveal permeable necrotic cells, 4′,6-diamidino-2-phenylindole (DAPI) to visualize apoptotic nuclei, and 3,3 dihexyloxacarbocyanine [DiOC6(3)][2,21] to monitor the loss of mitochondrial transmembrane potential revealed (Table 2) only a low induction of cell death by either compound even at 50 μM, the highest tested concentration (20% after exposure to **1**, 11% after exposure to **2**), largely accounted for by apoptosis (12.8% for **1**, 8.4% for **2**). Neither drug exerts a significant nonspecific toxicity on A-375 cells, thus suggesting that, as in the case of nevirapine,[5] the observed reduced cell proliferation is mainly due to the arrest or retardation of the cell cycle.

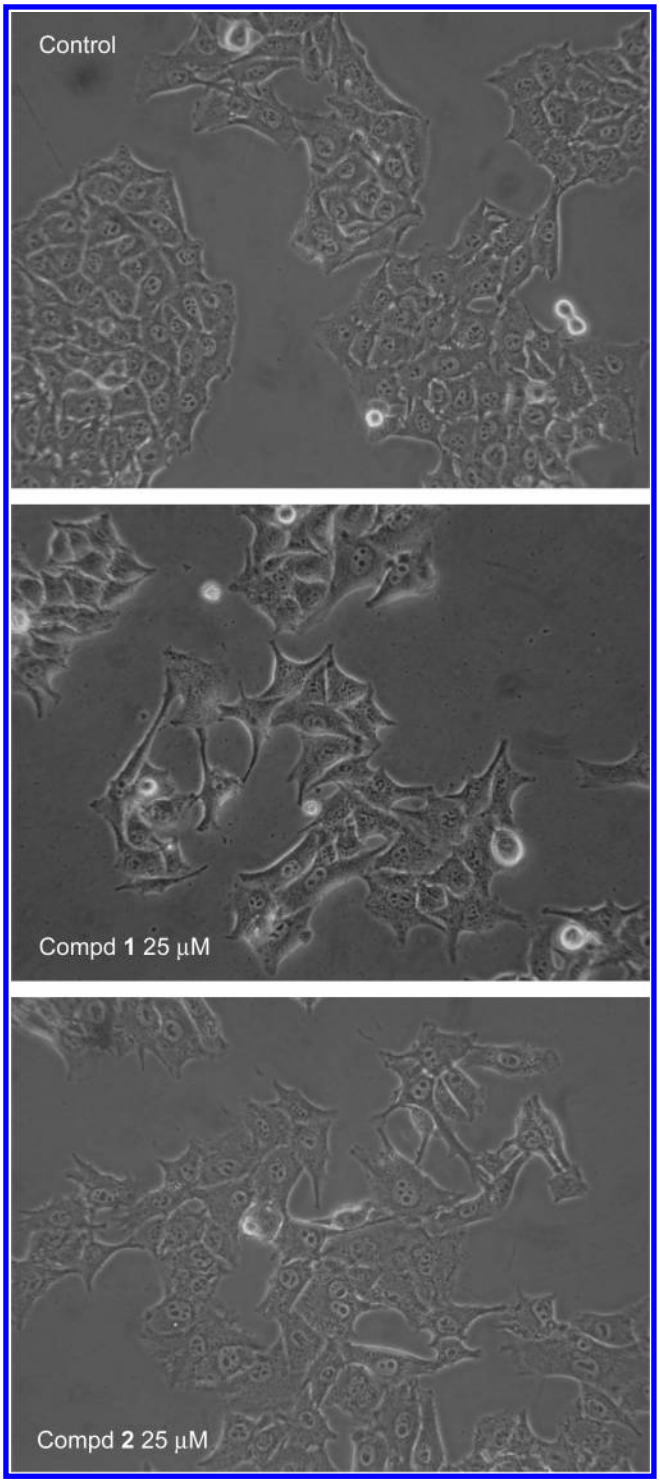

Figure 2. Morphological differentiation of A-375 melanoma cells in the presence of F_2-DABOs. Cultures were observed by phase-contrast microscopy.

Moreover, the modified cell shape, dendritic-like extensions, and increased adhesion in transformed cell lines (Figure 2) may suggest the occurrence of a dif-

ferentiation process, thus hinting[2] that critical regulatory genes are modulated in response to the RT-inhibitory treatment.

The effects induced by compounds **1** and **2** in the A-375 melanoma cell line closely resemble those previously observed in this cell line after exposure to chemical inhibitors of RT (nevirapine and efavirenz)[2,5] or to genetic inactivation of the RT-encoding LINE-1 elements by RNA interference (RNAi).[2] The similarity between the cellular phenotypes induced under all of these conditions with those presently reported for derivatives **1** and **2** are highly significant, indicating that a common activity is targeted in A-375 cells. Of note, these effects are induced relatively rapidly (in the order of days), which is a distinctive feature of RT inhibitors (nevirapine, efavirenz, and RNAi to LINE-1) and distinguishes them from chemical[22] or genetic[23] inhibition of the telomerase-associated RT. Taken together, these data support the idea that cellular alterations induced by **1** and **2** truly reflect the inhibition of the endogenous RT in A-375 cells.

In conclusion, the present work describes new data about the antiproliferative and cytodifferentiating activity of two compounds of the F_2-DABO class. In experiments with human differentiating cell systems, derivatives **1** and **2** significantly reduced cell proliferation and facilitated the morphological differentiation of cells. These results support the suggestion that NNRTIs could be useful tools in preventive and/or curative therapy to counteract the loss of differentiation in dedifferentiating pathologies and as antiproliferative drugs in tumor therapy. For the above reasons, F_2-DABOs are proposed as lead compounds for further research.

Acknowledgment. This work was supported by grants from PRIN 2004 and Istituto Superiore di Sanità (Italian National Institute of Health) (501/1 and 501/2).

Supporting Information Available: Experimental chemical and biological procedures and characterization data for compounds **1** and **2** and for intermediates **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Schramke, V.; Allshire, R. Hairpin. RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. *Science* **2003**, *301*, 1069−1074.
(2) Sciamanna, I.; Landriscina, M.; Pittoggi, C.; Quirino, M.; Mearelli, C.; Beraldi, R.; Mattei, E.; Serafino, A.; Cassano, A.; Sinibaldi-Vallebona, P.; Garaci, E.; Barone, C.; Spadafora, C. Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. *Oncogene* **2005**, *24*, 3923−3931 and references therein reported.
(3) Deininger, P. L.; Moran, J. V.; Batzer, M. A.; Kazazian, H. H. Mobile elements and mammalian genome evolution. *Curr. Opin. Genet. Dev.* **2003**, *13*, 651−658.
(4) Pyra, H.; Boni, J.; Schupbach, J. Ultrasensitive retrovirus detection by a reverse transcriptase assay based on product enhancement. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 1544−1548
(5) Mangiacasale, R.; Pittoggi, C.; Sciamanna, I.; Careddu, A.; Mattei, E.; Lorenzini, R.; Travaglini, L.; Landriscina, M.; Barone, C.; Nervi, C.; Lavia, P.; Spadafora, C. Exposure of normal and transformed cells to nevirapine, a Reverse Transcriptase inhibitor, reduces cell growth and promotes differentiation. *Oncogene* **2003**, *22*, 2750−2761.
(6) Pittoggi, C.; Sciamanna, I.; Mattei, E.; Beraldi, R.; Lobascio, A. M.; Mai, A.; Quaglia, M. G.; Lorenzini, R.; Spadafora, C. Role of endogenous reverse transcriptase in murine early embryo development. *Mol. Reprod. Dev.* **2003**, *66*, 225−236.
(7) Artico, M.; Massa, S.; Mai, A.; Marongiu, M. E.; Piras, G.; Tramontano, E.; La Colla, P. 3,4-Dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines (DABOs): a new class of specific inhibitors of human immunodeficiency virus type 1. *Antiviral Chem. Chemother.* **1993**, *4*, 361−368.
(8) Massa, S., Mai, A.; Artico, M.; Sbardella, G.; Tramontano, E.; Loi, A. G.; Scano, P.; La Colla, P. Synthesis and antiviral activity of new 3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines (DABOs), specific inhibitors of human immunodeficiency virus Type-1. *Antiviral Chem. Chemother*. **1995**, *6*, 1−8.
(9) Mai, A., Artico, M.; Sbardella, G.; Massa, S.; Loi, A. G.; Tramontano, E.; Scano, P.; La Colla, P. Synthesis and anti-HIV-1 activity of thio analogues of dihydroalkoxybenzyloxopyrimidines. *J. Med. Chem*. **1995**, *38*, 3258−3263.
(10) Mai, A.; Artico, M.; Sbardella, G.; Quartarone, S.; Massa, S.; Loi, A. G.; De Montis, A.; Scintu, F.; Putzolu, M.; La Colla, P. Dihydro(alkylthio)(naphthylmethyl) oxopyrimidines: novel non-nucleoside reverse transcriptase inhibitors of the *S*-DABO series. *J. Med. Chem.* **1997**, *40*, 1447−1454.
(11) Mai, A.; Artico, M.; Sbardella, G.; Massa, S.; Novellino, E.; Greco, G.; Loi A. G.; Tramontano, E.; Marongiu, M. E.; La Colla, P. 5-Alkyl-2-(alkylthio)-6-(2,6-dihalophenylmethyl)-3,4-dihydropyrimidin-4 (3*H*)-ones: novel potent and selective dihydro-alkoxybenzyl-oxopyrimidine derivatives. *J. Med. Chem.* **1999**, *42*, 619−627.
(12) Artico, M.; Mai, A.; Sbardella, G.; Massa, S.; Marceddu, T.; Vargiu, L.; Marongiu, M. E.; La Colla, P. Does the 2-methylthiomethyl substituent really confer high anti-HIV-1 activity to *S*-DABOs? *Med. Chem. Res.* **2000**, *10*, 30−39.
(13) Mai, A.; Sbardella, G.; Artico, M.; Ragno, R.; Massa, S.; Novellino, E.; Greco, G.; Lavecchia, A.; Musiu, C.; La Colla, M.; Murgioni, C.; La Colla, P.; Loddo, R. Structure-based design, synthesis, and biological evaluation of conformationally restricted novel 2-alkylthio-6-[1-(2,6-difluorophenyl) alkyl]-3,4-dihydro-5-alkylpyrimidin-4(3*H*)-ones as non-nucleoside inhibitors of HIV-1 reverse transcriptase. *J. Med. Chem.* **2001**, *44*, 2544−2554.
(14) Sbardella, G.; Mai, A.; Artico, M.; Chimenti, P.; Massa, S.; Loddo, R.; Marongiu, M. E.; La Colla, P.; Pani A. Structure−activity relationship studies on new DABOs: effect of substitutions at pyrimidine C-5 and C-6 positions on anti-HIV-1 activity. *Antiviral Chem. Chemother.* **2001**, *12*, 37−50.
(15) Pani, A.; Musiu, C.; Loi, A. G.; Mai, A.; Loddo, R.; La Colla, P.; Marongiu, M. E. DABOs as candidates to prevent mucosal HIV transmission *Antiviral Chem. Chemother.* **2001**, (Suppl. 1), 51−59.
(16) Ragno, R.; Mai, A.; Sbardella, G.; Artico, M.; Massa, S.; Musiu, C.; Mura, M.; Marceddu, T.; Cadeddu, A.; La Colla, P. Computer-aided design, synthesis, and anti-HIV-1 activity in vitro of 2-alkylamino-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4-(3*H*)-ones as novel potent non-nucleoside reverse transcriptase inhibitors, also active against the Y181C variant. *J. Med. Chem.* **2004**, *47*, 928−934.
(17) Mai, A.; Artico, M.; Ragno, R.; Sbardella, G.; Massa, S.; Musiu, C.; Mura, M.; Marturana, F.; Cadeddu, A.; Maga, G.; La Colla, P. 5-Alkyl-2-alkyl amino-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. *Bioorg. Med. Chem.* **2005**, *13*, 2065−2077.
(18) We decided to select for this screening the derivatives among the latest F_2-DABOs endowed with the best selectivity index (cytotoxic concentration/effective concentration ratio). Compounds **1** and **2** meet the requirements (references 13 and 15).
(19) As a first approach we decided to test only the two racemic forms. Biological assays for both the enantiomers of compound **1** as well as chiral resolution of racemic **2** are in progress.
(20) Maximum testable concentration for efavirenz in this condition was 15 μM.
(21) A very comprehensive review of experimental methods in cell death analysis can be found in Robinson, J. P. *The Purdue Cytometry CD-ROM*; Purdue University Cytometry Laboratories: West Lafayette, IN, 2000; Volume 5. ISBN 1-890473-05-7, http://flowcyt.cyto.purdue.edu/flowcyt/cdseries.htm.
(22) Damm, K.; Hemmann, U.; Garin-Chesa, P.; Hauel, N.; Kauffmann, I.; Priepke, H.; Niestroj, C.; Daiber, C.; Enenkel, B.; Guilliard, B.; Lauritsch, I.; Muller, E.; Pascolo, E.; Sauter, G.; Pantic, M.; Martens, U. M.; Wenz, C.; Lingner, J.; Kraut, N.; Rettig, W. J.; Schnapp, A. A highly selective telomerase inhibitor limiting human cancer cell proliferation. *EMBO J.* **2001**, *20*, 6958−68.
(23) Blasco, M. A.; Lee, H. W.; Hande, M. P.; Samper, E.; Lansdorp, P. M.; DePinho, R. A.; Greider, C. W. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **1997**, *91*, 25−34.

JM0507330