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6-Alkylthio-4-[1-(2,6-difluorophenyl)alkyl]-1*H*-[1,3,5]triazin-2-ones (ADATs): Novel Regulators of Cell Differentiation and Proliferation**

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Novel triazine analogues of 5-alkyl-2-alkylthio-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydropyrimidin-4(3H)-ones (F_2-DABOs), previously described by us as nonnucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs), were tested for their antiproliferative and cytodifferentiating activity on the A-375 human melanoma cell line. Most of the tested derivatives were effective in decreasing cell proliferation, facilitating morphological differentiation, and reprogramming gene expression. All these effects were reversible upon withdrawal of RT inhibitors. Among the compounds tested, ***3f*** *showed the highest antiproliferative effect, whereas compound* ***6c****, although not affecting cell proliferation, is endowed with a strong cytodifferentiating effect, which is probably related to a marked upregulation of the* e-cad *gene. These results support the potential of NNRTIs as valuable antitumor agents.*

Introduction

Retrotransposable elements, both retrotransposons (such as long interspersed nuclear element-1 (LINEs), Alu elements, and others) and endogenous retroviruses (HERVs), represent a significant portion of the human genome (45%). In contrast, protein-encoding genes represent a mere 1.2% of the total genome.[1,2] Some of these retrotransposable elements code for an endogenous reverse transcriptase (RT) activity,[2] which enables them to replicate and disperse in the host genome through an RNA intermediate.[3] Recent evidence supports the view that retrotransposons can destabilize and reshape the genome,[4] and that epigenetic modifications such as DNA methylation play a key role in the regulation of gene expression in mammals by keeping these movable and amplifiable elements transcriptionally repressed.[5,6]

It is well established that RT-coding genes are expressed at basal levels, if at all, in differentiated nonpathological tissues; in contrast, high expression is distinctive of embryonic (undifferentiated) and transformed (dedifferentiated) cells, suggesting that levels of RT expression are linked to the proliferative potential of the cell.[7] On these grounds, unscheduled activity of retrotransposons and HERVs is implicated in a variety of diseases.[5,8]

Studies developed in our research groups over the last years have shown that the commercially available RT inhibitors nevirapine and efavirenz, widely used in AIDS therapy, cause the arrest of early embryonic development[9] and are able to modulate cell growth and differentiation in a variety of cell lines by reducing proliferation, inducing morphological differentiation and reprogramming gene expression.[7,10–12] Discontinuation of anti-RT treatment reverts these features, 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 the aforementioned RT inhibitors attenuates the tumorigenic phenotype of prostate carcinoma cells inoculated in nude mice.[11] Based on these data, endogenous RT acts at an (as yet unknown) epigenetic level in cell transformation and tumor progression and may be a potential target for novel anticancer therapeutic approaches.

Inspired by this evidence, we recently investigated and described[13] the antiproliferative and cytodifferentiating activity of two compounds (**1** and **2**) of the F_2-DABO series (5-alkyl-2-alkylthio-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydropyrimidin-

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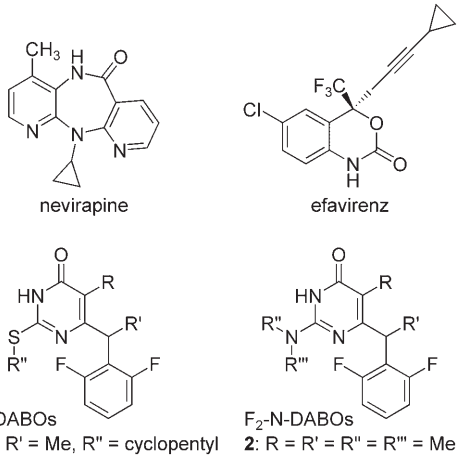
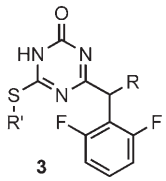
[**] *Previous papers in this series: "6-[1-(2,6-Difluorophenyl)ethyl]pyrimidinones Antagonize Cell Proliferation and Induce Cell Differentiation by Inhibiting (a Nontelomeric) Endogenous Reverse Transcriptase": S. Bartolini, A. Mai, M. Artico, N. Paesano, D. Rotili, C. Spadafora, G. Sbardella,* J. Med. Chem. ***2005***, 48, *6776–6778.*

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author: experimental procedures and characterization data for compounds **4b**–**d**, characterization data for the corresponding esters, elemental analysis of synthesized molecules, and oligonucleotide primer sequences used for PCR.

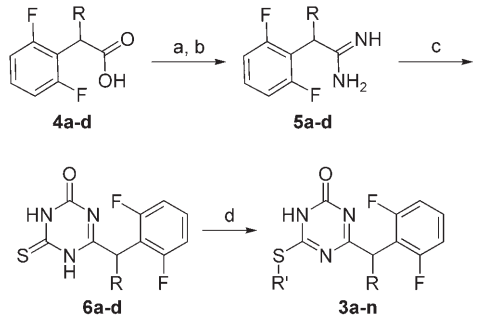


4(3*H*)-ones), developed by our group as nonnucleoside HIV-1 reverse transcriptase inhibitors (NNRTIs) after a decade of rational lead optimization studies.[14–24] In experiments with human differentiating cell systems, derivatives **1** and **2** significantly reduced cell proliferation and facilitated the morphological differentiation of human melanoma A-375 cells, thus supporting the suggestion that NNRTIs could be useful tools in preventive or curative therapy to counteract the loss of differentiation in dedifferentiating pathologies and as antiproliferative drugs in tumor therapy. Moreover, these results prompted us to choose F_2-DABOs as lead compounds for further research. Particularly, as reports have recently appeared of triazine-containing compounds as inducing the downregulation of human telomerase reverse transcriptase (hTERT)[25] and as a novel NNRTI class of anti-HIV-1 agents,[26] we were intrigued by the investigation of the effect on biological activity of replacing the substituted C5 of the pyrimidine ring with a nitrogen atom. Herein we report the synthesis and characterization of a novel series of 6-alkylthio-4-[1-(2,6-difluorophenyl)alkyl]-1*H*-[1,3,5]triazin-2-ones (ADATs, **3**), triazine analogues of F_2-S-DABOs, as antiproliferative and cytodifferentiating agents.



Chemistry

The title derivatives **3**[27] were synthesized starting from the reaction of ethoxycarbonylisothiocyanate[28] with 2-(2,6-difluorophenyl)alkanamidines **5**, prepared from the corresponding carboxylic acids **4**[29] by esterification followed with a Garigipati reaction[30] (Scheme 1). S-alkylation of the obtained 4-[1-(2,6-difluorophenyl)alkyl]-6-thioxo-5,6-dihydro-1*H*-[1,3,5]triazin-2-ones **6** with the appropriate alkyl iodide in the presence of potassium carbonate finally yielded the desired compounds.



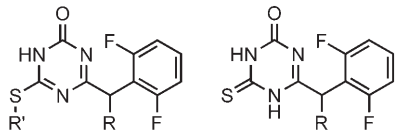
Scheme 1. Reagents and conditions: a) MeOH, H_2SO_4, reflux; b) NH_4Cl, $AlMe_3$/toluene, 0 °C then 80 °C; c) SCNCOOEt, NaOH (1 N), benzene/H_2O; d) R'I, K_2CO_3, DMF.

Results and Discussion

The antiproliferative activities of compounds **3a–n** and **6a–d** (in solutions of DMSO) were tested on human A-375 melanoma (ATCC CRL-1619) cell lines in comparison with **1**, nevirapine, and efavirenz as reference drugs.

Table 1 shows that even though with varying efficiency, most of the tested derivatives were effective in reducing cell

Table 1. Inhibition of proliferation by compounds **3a–n** and **6a–d**.[a]



Compd	R	R′	Cell Growth [%][b] [Compd] = 50 μM	[Compd] = 100 μM
control	–	–	100	
6a	H	–	100	92
3a	H	Me	100	97
3b	H	*i*Pr	95	56
3c	H	*c*Pen	62	30
6b	Me	–	98	90
3d	Me	Me	76	57
3e	Me	*i*Pr	83	42
3f	Me	*s*Bu	62	14
3g	Me	*c*Pen	56	43
3h	Me	Bn	51	43
6c	Et	–	98	90
3i	Et	Me	55	47
3j	Et	*i*Pr	80	20
3k	Et	*c*Pen	57	30
6d	*i*Pr	–	99	73
3l	*i*Pr	Me	54	31
3m	*i*Pr	*i*Pr	55	20
3n	*i*Pr	*c*Pen	65	31
1	–	–	14	9.5
NEV[c]	–	–	35	
EFV[d]	–	–	51	

[a] Cell growth in human A-375 melanoma cultures treated with DMSO (control), test compounds, nevirapine (NEV) or efavirenz (EFV). [b] Cells were harvested and counted after 96 h. Counted cells are expressed as percentage of control counts (set at 100%). Values represent the average of three experiments. [c] At 350 μM. [d] At 15 μM, the maximum testable concentration.

proliferation, as many of them were more effective than nevirapine at concentrations 3.5-fold lower (compare the efficacy of **3c**, **3f**, **3j**, **3k**, **3l**, **3m**, and **3n** at 100 μM with that of NEV at 350 μM). Replacement of the pyrimidine C5 of F_2-S-DABOs with a nitrogen atom (at the N3 position of the triazine nucleus) resulted in a general decrease in antiproliferative activity (compare the efficacy of derivative **1** with that of its ADAT counterpart **3g**).

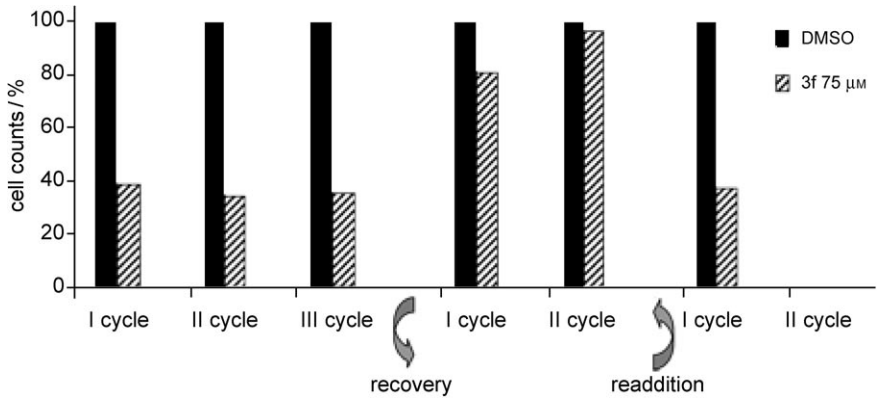


Figure 1. Inhibition of proliferation by compound **3f**: cells were grown in cultures treated with DMSO (control) or **3f**, and were counted and re-plated every 96 h for three cycles. Cells were then cultured in inhibitor-free medium (two cycles recovery). Compound **3f** was then re-added for two cycles. Counted cells are expressed as the percentage of control counts. Values represent the average of three experiments.

From the SAR analysis of data recorded in Table 1, it is clear that the sulfur atom at the C6 position of the triazine ring needs to be substituted by an alkyl group (R′) to confer antiproliferative activity against human A-375 cells, as the 6-thioxo[1,3,5]triazin-2-ones **6a–d** show very little (**6d** at 100 μM) or no (**6a–d** at 50 μM and **6a–c** at 100 μM) activity in this assay. If the substituent at the sulfur of C6 is a small- or, to a lesser extent, medium-sized alkyl (methyl or isopropyl) group, the extent to which cell proliferation is inhibited depends on the insertion of a further substituent (R) at the benzylic position of the C4-difluorobenzyl group. The 4-[2-methyl-1-(2,6-difluorophenyl)-1-propyl]-substituted analogues **3l** and **3m** are 2-fold (at 50 μM) to 3-fold (at 100 μM) more potent than the 4-(2,6-difluorobenzyl) counterparts **3a** and **3b**. In the 6-cyclopentylthio-substituted compounds **3c**, **3g**, **3k**, and **3n**, the insertion of a substituent at the C4 benzylic position did not influence the antiproliferative activity. The 4-(2,6-difluorobenzyl)triazine **3c** showed the same potency as the corresponding derivatives **3g**, **3k**, and **3n**, which respectively bear methyl, ethyl, isopropyl substituents at the benzylic position. Among the 4-[1-(2,6-difluorophenyl)-1-ethyl]triazines **3d–h** (with R = methyl at the benzylic position), **3f** showed the highest inhibitory effect toward human A-375 cells, with 86% cell growth inhibition at 100 μM.

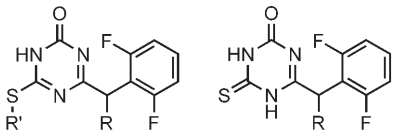
Several other ADAT derivatives such as **3j** and **3m** exhibited interesting activities, but we decided to further investigate the response of human transformed cell lines to prolonged exposure to compound **3f**, which is endowed with the most potent antiproliferative effect (Table 1). A-375 melanoma cells were counted and replated every 96 h with continuous addition of drug (or DMSO alone in control cultures) for at least three 96-hour cycles.

As shown in Figure 1, derivative **3f** effectively reduces cell proliferation, with a stable effect during prolonged exposure. Inhibition was reversible; when **3f** was removed from the cell culture medium, proliferation resumed at a rate similar to that of controls within two cycles. Return of the drug re-established proliferation inhibition in the cell line. Thus, the reduction of cell growth induced by RT inhibition is not inherited as a permanent change through cell division, which suggests that the phenotypic variations induced by RT inhibition in cultured cells are of an epigenetic nature.

We next explored whether the antiproliferative effect of tested compounds could be due to cell death. As shown in Table 2, differential cell staining with propidium iodide (PI) to reveal permeable necrotic cells, 4′,6-diamidino-2-phenylindole lactate (DAPI) to visualize apoptotic nuclei, and 3,3-dihexyloxacarbocyanine (DiOC6(3))[13,31] to monitor the loss of mitochondrial transmembrane potential revealed that: 1) all compounds induced a low level of cell death, even when cells were exposed to the highest tested concentration (100 μM) and 2) apoptosis accounted for most of the cell death (15.7% for **3f**). None of the compounds exerted any significant nonspecific toxicity toward A-375 cells, suggesting that, as in the case of nevirapine and F_2-DABOs **1** and **2**,[10,13] the observed decrease in cell proliferation is mainly due to the arrest or slowing of the cell cycle. Regarding the structure–activity relationships, we again observed a correlation between the extent of the apoptotic effect and the size of the substituent at the C4 benzylic position and at the C2-linked sulfur atom: the bulkier the substituent, the higher the level of apoptosis. Exceptions to this are the 6-cyclopentylthio derivatives **3c**, **3g**, **3k**, and **3n**, for which substitution at the benzylic position lowered the induction of apoptosis.

As melanomas are resistant to most therapeutic treatments, we thought it was relevant to determine whether ADAT derivatives could induce differentiation concomitant with reduced cell growth. As reported by our research group[11] and others,[32] the induction of differentiation in human melanoma cells causes a modified cell shape, characterized by dendritic-like extensions and increased adhesion. Therefore, evaluation of the changes in A-375 cell shape was used to assess the differentiating power of the different compounds (not shown). Unexpectedly, the highest degree of differentiation was effected by compound **6c**. A-375 cells exposed for 4–5 days to **6c** exhibit morphological alterations (Figure 2c) in comparison with DMSO-treated control cells (Figure 2a); such changes closely resemble those previously reported for nevirapine and F_2-DABOs **1** and **2**.[10,13]

Table 2. Effect of compounds **3a–n** and **6a–d** on cell death.[a]



3a-n **6a-d**

Compd	R	R′	[Compd] = 50 μM[b] Alive	Necrotic	Apoptotic	[Compd] = 100 μM[b] Alive	Necrotic	Apoptotic
control	–	–	98.3	0.4	1.3	98.3	0.4	1.3
6a	H	–	98.1	0.5	1.4	97	0.8	2.2
3a	H	Me	98.2	0.6	1.2	98	1	1
3b	H	*i*Pr	98.2	0.6	1.2	95.6	2	2.4
3c	H	*c*Pen	93.7	2.2	4.1	87	2	11
6b	Me	–	99.1	0.2	0.7	98.8	0.2	1
3d	Me	Me	97.8	0.6	1.6	96.8	0.8	2.4
3e	Me	*i*Pr	96.7	0.5	2.8	95.7	0.9	3.4
3f	Me	*s*Bu	94	2	4	78	6.3	15.7
3g	Me	*c*Pen	95.7	0.6	3.7	94.6	1.2	4.2
3h	Me	Bn	92.5	1.4	6.1	90	2	8
6c	Et	-	97	0.7	2.3	96.6	0.8	2.6
3i	Et	Me	94.2	1.6	4.2	94.5	1.9	3.6
3j	Et	*i*Pr	96.4	0.6	3	87	4.7	8.3
3k	Et	*c*Pen	94.6	0.9	4.5	88	3.4	8.6
6d	*i*Pr	–	97	0.6	2.4	96.4	0.9	2.7
3l	*i*Pr	Me	94.6	1.6	4.2	94	1.4	4.6
3m	*i*Pr	*i*Pr	96.4	0.6	3	83	5	12
3n	*i*Pr	*c*Pen	97.7	0.2	2	92	1.4	6.6

[a] Cell death in human A-375 melanoma cultures treated with DMSO (control) or test compounds for 96 h. [b] Counted cells are expressed as percentage of control counts (set at 100%). Values represent the average of three experiments.

This is particularly noteworthy because we observed no significant differentiating effects for both uracil and thymine F_2-S-DABO counterparts of **6c** (manuscript in preparation). In addition, immunofluorescence analysis using an anti-tubulin antibody also revealed the reorganization of the microtubule network throughout the length of outgrowing dendrites in cells inhibited with **6c** (Figure 2d), with the appearance of fusiform extensions protruding from the cell periphery. In contrast, DMSO-treated (control) cells show short microtubules that concentrate around the nucleating centers (Figure 2b). Again, the maintenance of differentiated features depends on the presence of RT inhibitors, and cells return to their original state within a few days of drug withdrawal.

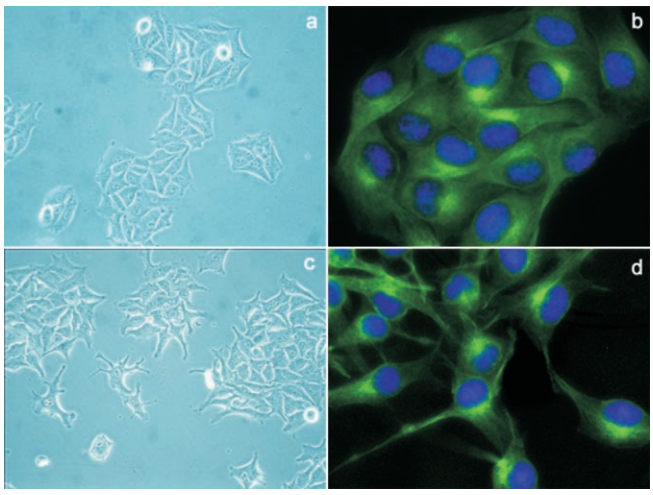


Figure 2. Morphological differentiation of A-375 melanoma cells in the presence of compounds **6c**. DMSO-treated (a and b) and 100 μM **6c**-treated (c and d) A-375 cells under phase-contrast microscopy (in a and c) and fluorescence microscopy (in b and d) after α-tubulin (green) and DAPI staining of nuclei (blue).

The induction of morphological differentiation suggests that critical regulatory genes are modulated in response to RT inhibitory treatment, as we already postulated in our preliminary study on compounds **1** and **2**.[13] Hence, we decided to investigate this feature in a semiquantitative RT-PCR analysis of cultures treated with DMSO alone (control), **6c**, **3f**, or **6a** for four cycles. In A-375 melanoma cells, we focused on a set of five genes: the *e-cadherin* (*e-cad*) gene, which is involved in cell–cell adhesion and is expressed in differentiated but not in tumor cells,[33] the oncosuppressor *p53* gene, which induces growth arrest or apoptotic cell death in various cell types depending on the particular growth environment,[34] *c-myc* and *cyclin D1* (*ccnd1*) genes,[35–37] which are directly implicated in the control of cell proliferation and tumor growth, and the antiapoptotic B cell lymphoma 2 (*bcl-2*) gene, which is overexpressed in many types of human cancers.[38] As a reference in our RT-PCR analysis, we used the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene, which is required for the maintenance of basal cellular function and is constitutively expressed in all human cells;[39] *gadph* expression is not altered by RT inhibition.

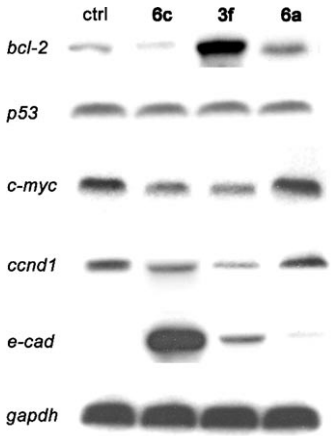


Figure 3. ADAT derivatives modulate gene expression in A-375 melanoma cells. RNA extracted from cells treated with DMSO (ctrl), **6c**, **3f**, or **6a** was amplified by RT-PCR, blotted, and hybridized with internal oligonucleotides specific for the genes indicated at left.

The results shown in Figure 3 indicate that all three ADAT derivatives are able to modulate gene expression with varied efficiency. Specifically, exposure to compound **6c** causes a striking upregulation of the *e-cad* gene relative to control, whereas *c-myc*, *bcl-2*, and *ccnd1* genes are consistently downregulated. Treatment with compound **3f** resulted in the downregulation of *c-myc* and *ccnd1* together with the upregulation (even if less evident than with **6c**) of *e-cad*. Surprisingly, *bcl-2* expression was significantly upregulated. Finally, derivative **6a** failed to modulate the expression of *c-myc*, *ccnd1*, or *e-cad* and elicited only a slight upregulation of the *bcl-2* gene. The expression of the "housekeeping" *gapdh* or apoptotic regulatory *p53* genes was consistently unaffected. Similar to what we observed in previously reported experiments,[10,11] this reprogramming is reversible and is abolished when RT inhibition is released.

Conclusions

The effects induced by ADAT derivatives in the A-375 melanoma cell line closely resemble that which we previously reported by either exposing this cell line to NNRTIs (nevirapine, efavirenz, or F_2-DABOs)[10,11,13] or by functional knock-out of the RT-encoding LINE-1 elements by RNA interference (RNAi).[11] As a whole, these data support the conclusion that all the molecules tested, including those reported herein, share a common target in A-375 cells. It is also notable that these effects are induced quite rapidly (within a few days), in contrast to chemical[40] or genetic[41] inhibition of the telomerase-associated RT which requires several months of continuous exposure. Taken together, these data further confirm our idea that cellular alterations induced by NNRTIs are a consequence of the inhibition of the endogenous RT in A-375 cells.

In conclusion, the work presented herein describes the antiproliferative and cytodifferentiating activity of a novel class of RT inhibitors, many of which are more effective than nevirapine at concentrations 3.5-fold lower. Similar to what we previously observed for nevirapine and efavirenz, the effects of tested compounds on cell growth and differentiation correlate well with gene reprogramming (downregulation of *c-myc* and *ccnd1* expression and upregulation of *e-cad* expression), with the exception of the unexpected significant upregulation of *bcl-2* expression observed in cells treated with **3f**. This result requires further investigation.

ADAT derivatives are less active than F_2-DABOs as antiproliferative agents, yet several of them effectively reduce cell proliferation. None of the compounds exert significant nonspecific toxicity which suggests that the experimental reduction of cytoproliferation is mainly due to the arrest or slowing of the cell cycle. Among the compounds tested, **3f** showed the highest inhibitory effect toward human A-375 cells, with 86% cell growth inhibition at 100 μM. This inhibition is reversible and is not inherited as a permanent change through cell division, suggesting that the phenotypic variations induced by RT inhibition in cultured cells are of an epigenetic nature.

Although **6c**, which is characterized by a 6-thioxotriazin-2-one structure and the presence of an ethyl substituent at the linker position, does not affect cell proliferation, it showed a strong cytodifferentiating effect, as substantiated by a marked upregulation of the *e-cad* gene. In the same assay, **6a**, the corresponding linker-unsubstituted analogue of **6c**, was essentially inactive. The latter evidence together with the above-mentioned structure–activity relationship (for antiproliferative effects as well as for apoptosis) suggest a crucial role for methyl and ethyl substituents at this position. These results confirm the view that endogenous RT acts at an epigenetic level and that small molecules that target this enzyme may be regarded as novel potential anticancer agents.

Experimental Section

Chemistry: Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. IR spectra (KBr) were recorded on a Shimadzu FTIR-8000 instrument. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker Avance 300 spectrometer; chemical shifts are reported in δ (ppm) relative to the internal reference tetramethylsilane. Electronic impact mass spectrometry (EIMS) was performed on a Finnigan LCQ DECA ThermoQuest instrument (San José, USA). 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 or by using a $KMnO_4$ alkaline solution. 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 ≈20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values. Reagents were purchased from Lancaster Synthesis (Milan, Italy) or Sigma–Aldrich (Milan, Italy). As a rule, samples prepared for physical and biological studies were dried in high vacuum over P_2O_5 for 20 h at T = 25–110 °C depending on the sample melting point.

General procedure for the preparation of 2-(2,6-difluorophenyl)-alkanamidinium hydrochlorides 5a–d; example: 2-(2,6-difluorophenyl)propionamidinium hydrochloride (5b). A stirred solution of 2-(2,6-difluorophenyl)propionic acid **4b** (1.50 g, 8.06 mmol)[29] in

anhydrous methanol (50 mL) was treated with a catalytic amount of sulfuric acid and held at reflux for 2 h. After cooling, the solvent was removed, and the residue was dissolved in water (100 mL) and extracted with AcOEt (3×50 mL). The organic layer was washed with a saturated solution of sodium bicarbonate (3×50 mL), brine (3×50 mL), dried, and concentrated to give the methyl-2-(2,6-difluorophenyl)propionate (1.61 g, quantitative yield) as a TLC-pure, light-yellow oil, which was directly used in the following step.[42] Trimethylaluminum (40.3 mmol, 5 equiv, 20.15 mL of a 2.0 M solution in toluene) was added dropwise to a magnetically stirred slurry of ammonium chloride (40.3 mmol, 5 equiv, 2.16 g) in 30 mL dry toluene at 0 °C under a nitrogen atmosphere, and the resulting mixture was warmed to room temperature for 2 h or until no more evolution of gas was observed. A solution of the previously prepared methyl-2-(2,6-difluorophenyl)propionate (1.61 g, 8.06 mmol) in 10 mL anhydrous toluene was then added, and the mixture was stirred at 80 °C overnight. It was then cooled to 0 °C, and methanol was added with consequent stirring for 1 h at room temperature. After filtration, the solid was washed with methanol several times, and the solution evaporated to dryness in vacuo to furnish **5b** as a brown residue, which was washed with methanol and then purified by crystallization from methanol/dichloromethane. Yield: 80%; MS (EI, 70 ev) *m/z*: 185; ^{1}H NMR (D_2O): δ = 1.56–1.58 (d, 3H, CH_3), 3.98–4.03 (m, 1H, CH), 7.07–7.12 (m, 2H, C3,5 Ar-H), 7.36–7.46 ppm (m, 1H, C4 Ar-H).

Amidinium hydrochlorides **5a**, **5c**, and **5d** were obtained by following the same procedure starting from the corresponding acid **4**.[29]

5a: Yield: 80%; MS (EI, 70 ev) *m/z*: 171; ^{1}H NMR (D_2O): δ = 3.51 (s, 2H, CH_2), 7.01–7.09 (m, 2H, C3,5 Ar-H), 7.20–7.26 ppm (m, 1H, C4 Ar-H).

5c: Yield: 85%; MS (EI, 70 ev) *m/z*: 199; ^{1}H NMR (D_2O): δ = 0.77–0.82 (t, 3H, CH_3), 1.83–1.88 (m, 1H, CH_2), 2.14–2.19 (m, 1H, CH_2), 3.89–4.07 (m, 1H, CH), 7.07–7.12 (m, 2H, C3,5 Ar-H), 7.36–7.46 ppm (m, 1H, C4 Ar-H).

5d: Yield: 96%; MS (EI, 70 ev) *m/z*: 213; ^{1}H NMR (D_2O): δ = 1.00–1.03 (d, 3H, CH_3), 1.18–1.20 (d, 3H, CH_3), 2.98–3.02 (m, 1H, CH), 4.14–4.17 (m, 1H, CH), 7.22–7.34 (m, 2H, C3,5 Ar-H), 7.64–7.67 ppm (m, 1H, C4 Ar-H).

General procedure for the preparation of 4-[1-(2,6-difluorophenyl)alkyl]-6-thioxo-5,6-dihydro-1*H*-[1,3,5]triazin-2-ones 6a–d; example: 4-[1-(2,6-difluorophenyl)propyl]-6-thioxo-5,6-dihydro-1*H*-[1,3,5]triazin-2-one (6c). A solution of ethoxycarbonylisothiocyanate (1.4 equiv, 2.37 mL, 20.10 mmol) in benzene (10 mL) and a solution of NaOH (1 N, 1 equiv, 14.36 mL) were added simultaneously over a period of 15 min to a vigorously stirred mixture of 2-(2,6-difluorophenyl)butyramidinium hydrochloride (**5c**) (3.37 g, 14.36 mmol) in water (10 mL) and benzene (40 mL). After the organic layer turned yellow, more 1 N NaOH (1 equiv, 14.36 mL) was added, and the resulting mixture was stirred at room temperature for 1 h. The organic layer was then separated and treated again with 1 N NaOH (14.36 mL). The combined colorless alkaline extracts were acidified with 2 N H_2SO_4 and extracted with ethyl acetate (3×40 mL). The organic phases were combined, washed with brine (3×50 mL), dried, and concentrated in vacuo to give a yellow solid which was crystallized from benzene/acetonitrile. Yield: 96%; mp: 198–199 °C; MS (EI, 70 ev) *m/z*: 283; ^{1}H NMR ([D_6]DMSO): δ = 0.81–0.86 (t, 3H, CH_3), 1.84–1.91 (m, 1H, CH_2), 2.12–2.51 (m, 1H, CH_2), 4.12–4.17 (m, 1H, CH), 7.09–7.14 (m, 2H, C3,5 Ar-H), 7.37–7.48 (m, 1H, C4 Ar-H), 12.70 (s, 1H, NH, exchangeable with D_2O), 13.38 ppm (br s, 1H, NH, exchangeable with D_2O).

4-[1-(2,6-Difluorophenyl)alkyl]-6-thioxo-5,6-dihydro-1*H*-[1,3,5]triazin-2-ones **6a**, **6b**, and **6d** were obtained by following the same procedure starting from the corresponding amidinium hydrochloride **5a**, **5b**, and **5d**.

6a: Yield: 65%; mp: >260 °C, ethanol; MS (EI, 70 ev) *m/z*: 255; ^{1}H NMR ([D_6]DMSO): δ = 3.96 (s, 2H, CH_2), 7.12–7.17 (m, 2H, C3,5 Ar-H), 7.38–7.50 (m, 1H, C4 Ar-H), 12.70 (s, 1H, NH, exchangeable with D_2O), 13.38 ppm (br s, 1H, NH, exchangeable with D_2O).

6b: Yield: 56%; mp: 240–241 °C, benzene/acetonitrile; MS (EI, 70 ev) *m/z*: 269; ^{1}H NMR ([D_6]DMSO): δ = 1.47–1.49 (d, 3H, CH_3), 4.28–4.35 (m, 1H, CH), 7.08–7.14 (m, 2H, C3,5 Ar-H), 7.37–7.48 (m, 1H, C4 Ar-H), 12.70 (s, 1H, NH, exchangeable with D_2O), 13.38 ppm (br s, 1H, NH, exchangeable with D_2O).

6d: Yield: 72%; mp: 174–175 °C, benzene/acetonitrile; MS (EI, 70 ev) *m/z*: 297; ^{1}H NMR ($CDCl_3$): δ = 0.86–0.88 (d, *J* = 6.6 Hz, 3H, CH_3), 1.10–1.12 (d, *J* = 6.5 Hz, 3H, CH_3), 2.82 (m, 1H, C*H*(CH_3)$_2$), 3.94–3.97 (d, *J* = 9.0 Hz, 1H, CH), 6.96–7.01 (m, 2H, C3,5 Ar-H), 7.29–7.39 (m, 1H, C4 Ar-H), 9.57 (br s, 1H, NH, exchangeable with D_2O), 9.73 ppm (br s, 1H, NH, exchangeable with D_2O).

General procedure for the preparation of 6-alkylthio-4-[1-(2,6-difluorophenyl)alkyl]-1*H*-[1,3,5]triazin-2-ones 3a-n; example: 4-(2,6-difluorobenzyl)-6-isopropylthio-1*H*-[1,3,5]-triazin-2-one (3b). Potassium carbonate (1 equiv, 0.54 g, 3.92 mmol) and 2-iodopropane (1 equiv, 3,92 mmol, 0,39 mL) were added to a solution of **6a** (1.00 g, 3.92 mmol) in anhydrous DMF (4 mL). The resulting mixture was stirred at room temperature overnight, then diluted with water (50 mL), and the aqueous phase was extracted with AcOEt (3×40 mL). The combined organic phases were washed with brine, dried, and concentrated in vacuo to give TLC-pure **3b** as a white solid, which was further purified by crystallization from acetonitrile/benzene. Yield: 85%; mp: 161–162 °C; MS (EI, 70 ev) *m/z*: 297; ^{1}H NMR ($CDCl_3$): δ = 1.55–1.57 (d, *J* = 6.9 Hz, 6H, 2×CH_3), 4.04–4.13 (m, 1H, CH), 4.30 (s, 2H, CH_2), 7.14–7.19 (m, 2H, C3,5 Ar-H), 7.48–7.58 (m, 1H, C4 Ar-H), 11.25 ppm (br s, 1H, NH, exchangeable with D_2O).

6-Alkylthio-4-[1-(2,6-difluorophenyl)alkyl]-1*H*-[1,3,5]triazin-2-ones **3a** and **3c**–**n** were obtained by following the same procedure starting from the corresponding 4-[1-(2,6-difluorophenyl)alkyl]-6-thioxo-5,6-dihydro-1*H*-[1,3,5]triazin-2-ones **6a**–**d** and the proper alkyl halide.

3a: Yield: 45%; mp: 210–211 °C, acetonitrile; MS (EI, 70 ev) *m/z*: 269; ^{1}H NMR ($CDCl_3$): δ = 2.49 (s, 3H, CH_3), 4.27 (s, 2H, CH_2), 7.06–7.11 (m, 2H, C3,5 Ar-H), 7.40–7.50 (m, 1H, C4 Ar-H), 10.55 ppm (br s, 1H, NH, exchangeable with D_2O).

3c: Yield: 55%; mp: 140–141 °C, cyclohexane; MS (EI, 70 ev) *m/z*: 323; ^{1}H NMR ($CDCl_3$): δ = 1.61–1.73 (m, 6H, cyclopentane), 2.16 (m, 2H, cyclopentane), 3.98–4.02 (m, 1H, SCH), 4.30 (s, 2H, CH_2), 6.85–6.90 (m, 2H, C3,5 Ar-H), 7.20–7.26 (m, 1H, C4 Ar-H), 10.15 ppm (br s, 1H, NH, exchangeable with D_2O).

3d: Yield: 61%; mp: 121–122 °C, cyclohexane; MS (EI, 70 ev) *m/z*: 283; ^{1}H NMR ([D_6]DMSO): δ = 1.45–1.51 (d, *J* = 7.1 Hz, 3H, CH_3), 2.32 (s, 3H, SCH_3), 3.91–4.00 (q, *J* = 7.1 Hz, 1H, CH), 7.06–7.12 (m, 2H, C3,5 Ar-H), 7.35–7.45 (m, 1H, C4 Ar-H), 12.71 ppm (br s, 1H, NH, exchangeable with D_2O).

3e: Yield: 68%; mp: 158–160 °C, benzene; MS (EI, 70 ev) *m/z*: 311; ^{1}H NMR ($CDCl_3$): δ = 1.22–1.25 (d, *J* = 6.8 Hz, 3H, CH_3), 1.31–1.33 (d, *J* = 6.9 Hz, 3H, CH_3), 1.38–1.40 (d, *J* = 6.9 Hz, 3H, CH_3), 3.75–3.95 (m, 2H, CH + SCH), 6.87–6.92 (m, 2H, C3,5 Ar-H), 7.26–7.33 (m, 1H, C4 Ar-H), 10.21 ppm (br s, 1H, NH, exchangeable with D_2O).

3f: Yield: 72%; mp: 104–105 °C, cyclohexane; MS (EI, 70 ev) *m/z*: 325; ^{1}H NMR ($CDCl_3$): $\delta = 0.97–1.02$ (t, $J = 7.3$ Hz, 3H, CH_2CH_3), 1.35–1.37 (d, $J = 6.9$ Hz, 3H, CH_3), 1.60–1.74 (m, 2H, CH_2CH_3, overlapped signals), 1.72–1.74 (d, $J = 7.2$ Hz, 3H, CH_3), 3.76–3.84 (m, 1H, SCH), 4.41–4.48 (q, $J = 7.2$ Hz, 1H, CH), 6.91–6.97 (m, 2H, C3,5 Ar-H), 7.27–7.38 (m, 1H, C4 Ar-H), 10.18 ppm (br s, 1H, NH, exchangeable with D_2O).

3g: Yield: 43%; mp: 184–185 °C, acetonitrile; MS (EI, 70 ev) *m/z*: 337; ^{1}H NMR ([D_6]DMSO): $\delta = 1.50–1.52$ (d, $J = 7.1$ Hz, 3H, CH_3), 1.47–1.65 (m, 6H, cyclopentane, overlapped signals), 2.01–2.04 (m, 2H, cyclopentane), 3.74–3.81 (m, 1H, SCH), 4.35–4.42 (q, $J = 7.1$ Hz, 1H, CH), 7.06–7.12 (m, 2H, C3,5 Ar-H), 7.35–7.45 (m, 1H, C4 Ar-H), 12.71 ppm (br s, 1H, NH, exchangeable with D_2O).

3h: Yield: 63%; mp: 158–159 °C, benzene; MS (EI, 70 ev) *m/z*: 359; ^{1}H NMR ($CDCl_3$): $\delta = 1.60–1.63$ (d, $J = 7.1$ Hz, 3H, CH_3), 4.19 (s, 2H, CH_2), 4.31–4.38 (q, $J = 7.1$ Hz, 1H, CH), 6.79–6.84 (m, 2H, C3,5 Ar-H), 7.14–7.25 (m, 6H, C4 Ar-H and Ph, overlapped signals), 10.09 ppm (br s, 1H, NH, exchangeable with D_2O).

3i: Yield: 73%; mp: 152–153 °C, acetonitrile; MS (EI, 70 ev) *m/z*: 297; ^{1}H NMR ([D_6]DMSO): $\delta = 0.80–0.85$ (t, 3H, CH_3), 1.82–1.92 (m, 1H, CH_2), 2.17–2.28 (m, 1H, CH_2), 2.40 (s, 3H, SCH_3), 4.17–4.22 (m, 1H, CH), 7.08–7.14 (m, 2H, C3,5 Ar-H), 7.37–7.48 (m, 1H, C4 Ar-H), 12.69 ppm (br s, 1H, NH, exchangeable with D_2O).

3j: Yield: 65%; mp: 173–174 °C, acetonitrile; MS (EI, 70 ev) *m/z*: 325; ^{1}H NMR ([D_6]DMSO): $\delta = 0.80–0.85$ (t, 3H, CH_3), 1.31–1.33 (d, $J = 6.9$ Hz, 3H, CH_3), 1.38–1.40 (d, $J = 6.9$ Hz, 3H, CH_3), 1.82–1.92 (m, 1H, CH_2), 2.17–2.28 (m, 1H, CH_2), 3.76–3.84 (m, 1H, SCH), 4.17–4.22 (m, 1H, CH), 7.08–7.13 (m, 2H, C3,5 Ar-H), 7.38–7.48 (m, 1H, C4 Ar-H), 12.67 ppm (br s, 1H, NH, exchangeable with D_2O).

3k: Yield: 35%; mp: 104–105 °C, cyclohexane; MS (EI, 70 ev) *m/z*: 351; ^{1}H NMR ([D_6]DMSO): $\delta = 0.81–0.86$ (t, 3H, CH_3), 1.53–1.66 (m, 6H, cyclopentane), 1.84–2.03 (m, 3H, CH_2 and cyclopentane, overlapped signals), 2.20–2.28 (m, 1H, CH_2), 3.75–3.82 (m, 1H, SCH), 4.17–4.22 (m, 1H, CH), 7.08–7.14 (m, 2H, C3,5 Ar-H), 7.37–7.48 (m, 1H, C4 Ar-H), 12.73 ppm (br s, 1H, NH, exchangeable with D_2O).

3l: Yield: 69%; mp: 159–161 °C, benzene; MS (EI, 70 ev) *m/z*: 311; ^{1}H NMR ([D_6]DMSO): $\delta = 0.88–0.90$ (d, $J = 6.6$ Hz, 3H, CH_3), 1.06–1.08 (d, $J = 6.5$ Hz, 3H, CH_3), 2.19 (s, 3H, SCH_3), 2.83–2.91 (m, 1H, $CH(CH_3)_2$), 4.02–4.05 (d, $J = 9.0$ Hz, 1H, CH), 7.16–7.22 (m, 2H, C3,5 Ar-H), 7.45–7.55 (m, 1H, C4 Ar-H), 12.82 ppm (br s, 1H, NH, exchangeable with D_2O).

3m: Yield: 61%; mp: 147–148 °C, benzene; MS (EI, 70 ev) *m/z*: 339; ^{1}H NMR ($CDCl_3$): $\delta = 0.86–0.88$ (d, $J = 6.7$ Hz, 3H, CH_3), 1.06–1.08 (d, $J = 6.5$ Hz, 3H, CH_3), 1.37–1.45 (m, 6H, $2\times CH_3$), 2.84 (m, 1H, $CH(CH_3)_2$), 3.92–3.99 (m, 2H, CH + SCH), 6.90–6.96 (m, 2H, C3,5 Ar-H), 7.26–7.30 (m, 1H, C4 Ar-H), 10.21 ppm (br s, 1H, NH, exchangeable with D_2O).

3n: Yield: 51%; mp: 98–99 °C, benzene/cyclohexane; MS (EI, 70 ev) *m/z*: 365; ^{1}H NMR ($CDCl_3$): $\delta = 0.86–1.02$ (d, $J = 6.5$ Hz, 3H, CH_3), 1.04–1.06 (d, $J = 6.4$ Hz, 3H, CH_3), 1.61–1.73 (m, 6H, cyclopentane), 2.16 (m, 2H, cyclopentane), 2.91 (m, 1H, $CH(CH_3)_2$), 3.93–4.02 (m, 2H, CH + SCH), 6.85–6.90 (m, 2H, C3,5 Ar-H), 7.20–7.26 (m, 1H, C4 Ar-H), 10.15 (br s, 1H, NH, exchangeable with D_2O).

Cell cultures: Human A-375 melanoma (ATCC-CRL-1619) cells were seeded in six-well plates at a density of 1×10^4–5×10^4 cells per well and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone) with 10% fetal bovine serum. Nevirapine and efavirenz were purified from commercially available Viramune (Boehringer Ingelheim) and Sustiva (Bristol-Myers Squibb) as described.[9] Compounds **3a**–**n**, **6a**–**d**, **1**, and the reference drugs nevirapine and efavirenz were solubilized in DMSO to various concentrations and were added to cells 5 h after seeding; the same DMSO volume (0.2% final concentration) was added to controls. Fresh RT-inhibitor-containing medium was changed every 48 h. Cells were harvested after 96 h, counted in a Burker chamber (two countings per sample) and replated at the same density.

Death analysis: Cell death was assessed by microscopy[11] after combined staining with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, nuclear morphology), propidium iodide (PI, Sigma, cell permeability), and 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3), Molecular Probes, a fluorescent probe for mitochondrial transmembrane potential). Cells were counted in a Burker chamber (two countings per sample).

Indirect immunofluorescence and confocal microscopy: Cell preparations were fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. Mouse monoclonal anti-bovine α-tubulin (Molecular Probes, A-11126) was revealed by Alexa-Fluor-488-conjugated secondary antibody (Molecular Probes, A-11001) in A-375 cells. Nuclei were stained either with 2 μg mL^{-1} PI in the presence of 0.1 μg mL^{-1} ribonuclease A or with 0.1 μg mL^{-1} DAPI. Samples were imaged under a confocal Leica TCS 4D microscope equipped with an Ar/Kr laser. Confocal sections were taken at intervals of 0.5–1 μm.

Semiquantitative RT-PCR: RNA extraction and treatment with RNase-free DNase I were carried out as described previously.[9] cDNAs were synthesized using 300 ng RNA, oligo-(dT) and the Thermoscript system (Invitrogen). Reaction mixtures (1:25) were amplified using the Platinum Taq DNA Polymerase kit (Invitrogen) and 30 pmol oligonucleotides (MWGBiotech, Ebersberg, Germany; see Supporting Information) in an initial 2-min step at 94 °C, followed by cycles of 30 s at 94 °C, 30 s at 58–62 °C, 1 min at 72 °C. Each oligonucleotide pair was used in sequential amplification series with increasing numbers (25–40) of cycles. PCR products were electrophoresed, transferred to membranes, and hybridized for 16 h at 42 °C with γ-[^{32}P]-ATP end-labeled internal oligonucleotides. The intensity of the amplification signal was measured by densitometry in at least three independent experiments for each gene and normalized to the GAPDH signal in the same experiment.

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[42] Characterization data are available in the Supporting Information.

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