Supporting Information

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

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Experimental procedures

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Infrared (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) units relative to the internal reference tetramethylsilane (Me_4Si). Electronic impact mass spectrometry (EI-MS) was performed on a LCQ DECA TermoQuest (San Josè, California, USA) mass spectrometer. All compounds were routinely checked by TLC and ^{1}H-NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values. (2,6-difluoro)phenylacetic acid were purchased from Lancaster Synthesis, thionyl chloride, *n*-butyl lithium 2.5 M solution in hexane, iodomethane, cyclopentyl bromide and 1,1-dimethylguanidine sulphate were purchased from Aldrich.

As a rule, samples prepared for physical and biological studies were dried in high vacuum over P_2O_5 for 20 h at temperatures ranging from 25 to 110 °C, depending on the sample melting point.

Preparation of 2-(2,6-difluoro)phenylpropionic acid (4).
To a a cooled (-10 °C) stirred solution of (2,6-Difluorophenyl)acetic acid (5.00 g, 29.05 mmol) in anhydrous THF (80 mL) 1.0 eq of *n*-butyl lithium 2.5 M solution in hexane (11.62 mL, 29.05 mmol) was added dropwise. After stirring for 15 min another 1.20 eq of *n*-butyl lithium 2.5 M solution in hexane (13.95 mL, 34.86 mmol) was added dropwise. The resulting mixture was stirred at room temperature for 1h, then it was recooled (-10 °C), treated with 1.2 eq of neat methyl iodide (2.18 mL, 34.86 mmol), and stirred at room temperature for 15 min. The clear solution was then poured into cold water (200 mL) and 2 N HCl (20 mL) and extracted with ethyl acetate (3 × 50 mL). The organic layers were collected, washed with sodium thiosulphate saturated solution (3 × 30 mL) and then with brine (3 × 50 mL), dried, and evaporated to furnish a residue that was purified by column chromatography (silica gel, ethyl acetate/chloroform 1/1). Yield 74% (mp: 51-54 °C, *n*-hexane).[*] MS (EI, 70 ev) m/z: 186; ^{1}H NMR ($CDCl_3$): δ 1.49-1.52 (d, 3H, CH_3), 4.07-4.18 (q, 1H, CH), 6.82-6.90 (m, 2H, C-$_{3,5}$ Ar-H), 7.15-7.24 (m, 1H, C-$_4$ Ar-H), 7.95 (s, 1H, OH exchangeable with D_2O). IR: 3060, 1700 cm^{-1}. Anal. calcd for $C_9H_8F_2O_2$: C, 58.07; H, 4.33; F, 20.41; Found C, 57.90, H, 4.29, F, 20.31.

Preparation of ethyl 4-(2,6-difluoro)phenyl-2-methyl-3-oxopentanoate (5)
Triethylamine (9.52 mL, 68.26 mmol) and magnesium chloride (5.13 g, 53.33 mmol) were added to a stirred suspension of potassium ethyl 2-methylmalonate (8.25 g, 44.79 mmol) in dry acetonitrile (60 mL), and stirring was continued at room temperature for 2 h. Then, a solution of the 2-(2,6-difluoro)phenylpropionyl chloride in dry acetonitrile (30 mL) was added dropwise. The latter solution was freshly prepared by refluxing **4** (3.97 g, 21.33 mmol) and thionyl chloride (10.00 mL, 106.65 mmol) for 2 h, evaporation of exceeding thionyl chloride under reduced pressure and resuspending the residue in dry acetonitrile (30 mL). The reaction was heated at reflux for 2 h. After the mixture had cooled, 13% HCl (50 mL) was cautiously added while keeping the temperature below 25 °C, and the resulting clear mixture was stirred for a further 15 min. The organic layer was separated and evaporated; then the residue was treated with ethyl acetate (50 mL). The aqueous layer was extracted with ethyl acetate (2 × 50 mL), and the organic phases were combined, washed with saturated sodium bicarbonate solution (2 × 100 mL) and brine (100 mL), dried, and concentrated to give **5** (92%) as a yellow oily mixture of tautomers (NMR), which was directly used in the following step[†]. MS (EI, 70 ev) m/z: 270; ^{1}H NMR ($CDCl_3$) δ 1.23-1.38 (m, 6H, COCH(C*H*$_3$)CO and CH_2CH_3), 1.46-1.64 (m, 3H, ArCHC*H*$_3$), 3.54-3.64 (m, 1H, COCHCO),

[*] We previously (reference 13) reported intermediate **4** as a yellow oil. Actually we later found that it is a low melting solid.
[†] In a previous work (reference 13) we separated (and described) the keto form of **5** from its enolic tautomers by LC; later on we found that all of them furnished the same condensation product in the following synthetic step (data not shown). For this reason we decided to use the crude mixture.

3.90-4.26 (m, 3H, ArC*H*CH_3 and C$H_2$$CH_3$), 6.88-6.99 (m, 2H, $C_{3,5}$-H Ar), 7.24-7.33 (m, 1H, C_4-H Ar); IR: 1725, 1700 cm^{-1}. Anal. calcd for $C_{14}H_{16}F_2O_3$: C, 62.22; H, 5.97; F, 14.06; Found C, 62.04, H, 5.94, F, 13.98.

Preparation of 6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methyl-2-thioxopyrimidin-4(3H)-one

Sodium metal (0.60 g, 26.2 mmol) was dissolved in 50 mL of absolute ethanol, and thiourea (1.39 g, 18.2 mmol) and **5** (3.53 g, 13.1 mmol) were added to the clear solution. The mixture was heated at reflux for 5 h. After the mixture was cooled, the solvent was distilled in vacuo at 40-50 °C until dry and the residue was dissolved in a little water (20 mL) and made acidic with 2 N HCl. The resulting precipitate was filtered under reduced pressure, washed with diethyl ether, and vacuum-dried at 80 °C for 12 h to give title compound as a pure solid which was further purified by crystallization (acetonitrile; mp 267–269 °C) (Yield: 40%). MS (EI, 70 ev) m/z: 282; ^{1}H NMR (DMSO-d_6): δ 1.82-1.91 (d, 3H, CHCH_3), 1.83 (s, 3H, C_5-CH_3, overlapped signal), 4.68-4.76 (q, 1H, CH-Ar), 7.31-7.36 (m, 2H, $C_{3,5}$-H Ar), 7.58-7.67 (m, 1H, C_4-H Ar), 11.86 (s, 1H, NH exchanged with D_2O), 12.66 (s, 1H, NH exchanged with D_2O). IR: 3100, 1630 cm^{-1}. Anal. calcd for $C_{13}H_{12}F_2N_2OS$: C, 55.31; H, 4.28; F, 13.46; N, 9.92; S, 11.36; Found C, 55.14, H, 4.25, F, 13.48, N, 9.90; S, 11.31.

Preparation of 2-cyclopentylthio-6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methylpyrimidin-4(3H)-one (1)

A mixture of 6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-5-methyl-2-thioxopyrimidin-4(3H)-one (0.48 g, 1.70 mmol), cyclopentyl bromide (0.27 g, 0.19 ml, 1.80 mmol), and potassium carbonate (0.23 g, 1.70 mmol) in 2 ml of anhydrous N,N-dimethylformamide was stirred at room temperature for 8 h. The reaction content was poured on cold water (100 ml) and extracted with ethyl acetate (3 × 50ml). The organic layers were collected, washed with brine (3 × 50 ml), dried overnight, then evaporated to furnish crude **1** as a solid, which was purified by crystallization (cyclohexane; mp 196–197°C). Yield: 60%. MS (EI, 70 ev) m/z: 350; ^{1}H NMR ($CDCl_3$) δ 1.43–1.67 (m, 9H, $C_{2,3,4,5}$-H cyclopentyl and CHCH_3), 1.99–2.17 (m, 5H, $C_{2,5}$-H cyclopentyl and C_5-CH_3), 3.98–4.02 (m, 1H, C_1-H cyclopentyl), 4.55–4.59 (q, 1H, C*H*CH_3), 6.77–6.85 (m, 2H, $C_{3,5}$-H Ar), 7.14–7.22 (m, 1H, C_4-H Ar), 11.88 (bs, 1H, NH exchangeable with D_2O). IR: 2900, 1640 cm^{-1}. Anal. calcd for $C_{18}H_{20}F_2N_2OS$: C, 61.69; H, 5.75; F, 10.84; N, 7.99; S, 9.15; Found C, 61.87, H, 5.78, F, 10.82, N, 8.01; S, 9.14.

Preparation of 6-[1-(2,6-difluorophenyl)ethyl]-3,4-dihydro-2-dimethylamino-5-methylpyrimidin-4(3H)-one (2).

Sodium metal (3 eq, 0.68 g, 29.75 mmol) was dissolved in 100 mL of absolute ethanol, and 1,1-dimethylguanidine sulphate (2.70 g, 9.92 mmol) and **5** (2.68 g, 9.92 mmol) were added to the clear solution. The mixture was heated at reflux for 5 h. After cooling, the solvent was distilled in vacuo at 40–50 °C until dry and the residue was dissolved in a little water (20 mL), made neutral (pH $\cong$ 7) with 1 N HCl, and extracted with ethyl acetate (3 × 50ml). The organic layers were collected, washed with brine (3 × 50 ml), dried overnight, then evaporated to furnish crude **2** which was purified by column chromatography (silica gel, ethyl acetate/chloroform 1/1). Yield 73% (ciclohexane; mp 174–175 °C). MS (EI, 70 ev) m/z: 293; ^{1}H NMR (DMSO-d_6): d 1.65–1.66 (d, 3H, ArCHCH_3), 1, 92 (s, 3H, C_5-CH_3), 3.11 (s, 6H, NCH_3), 4.53–4.58 (q, 1H, ArC*H*CH_3), 6.77–6.85 (m, 2H, $C_{3,5}$-H Ar), 7.12–7.20 (m, 1H, C_4-Ar–H). Anal. calcd for $C_{15}H_{17}F_2N_3O$: C, 61.42; H, 5.84; F, 12.95; N, 14.33; Found C, 61.56, H, 5.87, F, 12.93, N, 14.29.

Cell cultures

Human A-375 melanoma (ATCC-CRL-1619) cell lines were seeded in six-well plates at a density of 10^4 to 5 x 10^4 cells/well and cultured in DMEM (Euroclone) medium with 10% fetal bovine serum. Nevirapine and efavirenz were purified from commercially available Viramune (Boehringer-Ingelheim) and Sustiva (Bristol-Myers Squibb) as described (reference 6). Compounds **1** and **2** as well as reference drugs nevirapine and efavirenz were solubilized in dimethyl sulfoxide to obtain different concentrations (DMSO, Aldrich), and added to cells 5 h after seeding; the same DMSO volume (0.2% final concentration) was added to controls. Cells were harvested after 96 h and counted in a Burker chamber (two countings/sample).

Death analysis

Cell death was assessed by microscopy (Reference 2) after combined staining with 4',6-diamidino-2-phenylindole (DAPI, nuclear morphology, Sigma); propidium iodide (PI, cell permeability, Sigma); 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/sample).

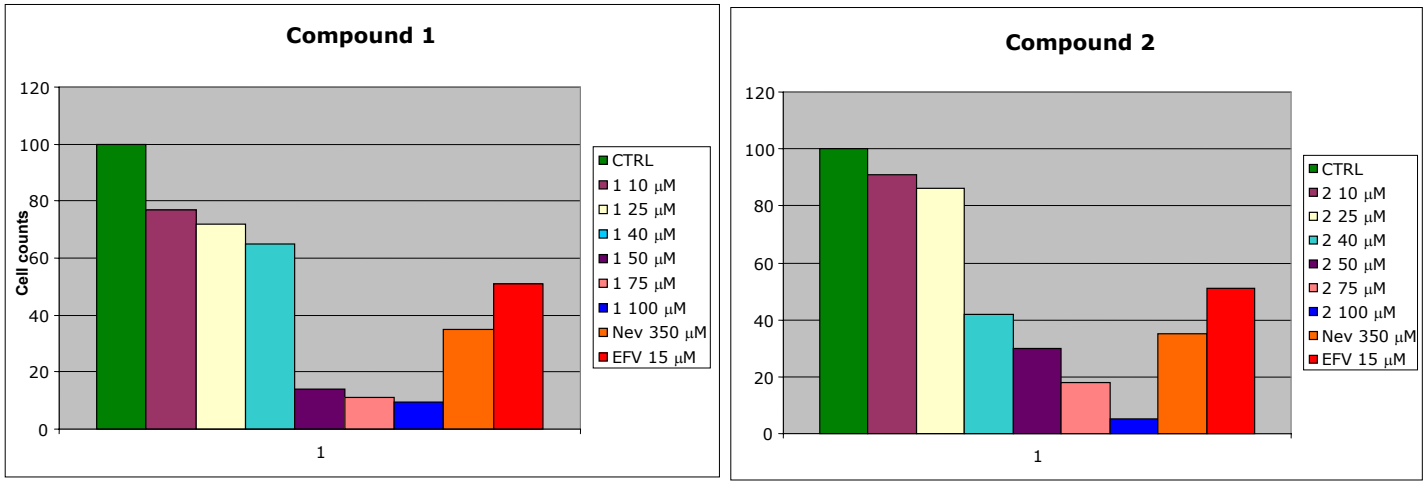

Figure 3. Inhibition of proliferation by F_2-DABOs. (A) Cell growth in human A-375 melanoma cultures treated with DMSO (control), **1**, nevirapine (NEV) and efavirenz (EFV). (B) Cell growth in cultures treated with DMSO (control), **2**, nevirapine (NEV) and efavirenz (EFV). Cells were harvested and counted after 96 h. Counted cells are expressed as the % of controls, taken as 100. Values represent pooled data from three experiments.

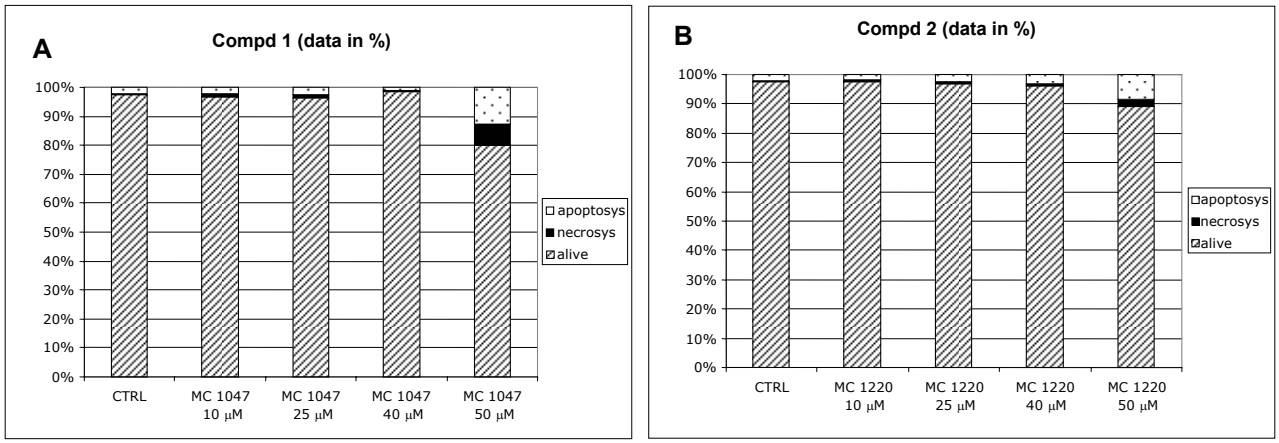

Figure 4. Effect of F_2-DABOs on cell death. (a) Human A-375 melanoma cultures treated with DMSO (control) and **1** for 96 h. (b) Human A-375 melanoma cultures treated with DMSO (control) and **2** for 96 h. Counted cells are expressed as the % of controls, taken as 100. Values represent pooled data from three experiments.