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Electronic Supporting Information

to the manuscript:

Trifluoromethyl derivatives of canonical nucleosides: synthesis and bioactivity studies

by

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Experimental Section.

General Methods.

All the reagents were of the highest commercially available quality and were used as received. TLC analyses were carried out on silica gel plates from Merck (60, F254). Reaction products on TLC plates were visualized by UV light and then by treatment with a 10 % $Ce(SO_4)_2/H_2SO_4$ aq. solution. For column chromatography, silica gel from Merck (Kieselgel 40, 0.063-0.200 mm) was used. The following abbreviations were used throughout the text: Ac = acetyl; AcOEt = ethyl acetate; DMAP = 4-dimethylaminopyridine; DMSO = dimethylsulfoxide; Et_2O = diethyl ether; TEA = triethylamine.

^1H and ^{13}C NMR spectra were recorded on Varian XR 200 and Varian Inova 500 spectrometers, as specified. All the chemical shifts are expressed in ppm with respect to the residual solvent signal. (for ^1H NMR: $CDCl_3$ = 7.26 ppm; CD_3OD = 3.31 ppm; for ^{13}C NMR: $CDCl_3$ = 76.9 ppm; CD_3OD = 49.5 ppm). Multiplicities are expressed with the following abbreviations: s = singlet; d = doublet; dd = double doublet; t = triplet; m = multiplet. Peak assignments have been carried out on the basis of standard ^1H-^1H COSY and HSQC experiments. ^{19}F NMR spectra were recorded on a Bruker 400 spectrometer at 376 MHz, using CF_3CD_2OD as internal standard. For the ESI MS analyses, a Waters Micromass ZQ instrument – equipped with an Electrospray source – was used in the positive mode.

Trifluoromethylation of 2'-deoxycytidine.

To a solution of 2'-deoxycytidine (50 mg, 0.22 mmol, 1 eq) in 1 ml of H_2O, CF_3SO_2Na (103 mg, 0.66 mmol, 3 eq) previously dissolved in H_2O (1 ml) and, successively, a 70% aq. solution of *tert*-BuOOH (105 µl, 1.10 mmol, 5 eq) were added at 0 °C. The reaction was then taken to r.t. After 3 h, the reaction was quenched by addition of a satd. aq. solution of $NaHCO_3$ (3 ml) and the resulting mixture taken to dryness. The crude was purified on a silica gel column eluted with a gradient from 5 to 15 % of CH_3OH in CH_2Cl_2, giving 47 mg of the target 5-CF_3-2'-deoxycytidine **2** (0.16 mmol, 73 % yield) and 12 mg (24 %) of the starting material.

2: R_f = 0.7 in 8:2 CH_2Cl_2/CH_3OH (v/v). **^{1}H NMR** (200 MHz, CD_3OD): δ 8.87 [s, 1H, H-6]; 6.20 [t, 1H, *J* = 5.8 Hz, H-1']; 4.39 [m, 1H, H-3']; 3.98 [m, 1H, H_a-5']; 3.81 [m, 1H, H-4']; 3.32 [m, 1H, H_b-5']; 2.44 [m, 1H, H_a-2']; 2.26 [m, 1H, H_b-2']. **^{13}C NMR** (50 MHz, CD_3OD): δ 162.6 [C-4]; 157.2 [C-2]; 145.4 [C-6]; 125.0 [q, *J* = 267.5 Hz, CF_3]; 98.5 [q, *J* = 35.4 Hz, C-5]; 89.7 [C-4']; 88.8 [C-1']; 71.6 [C-3']; 62.3 [C-5']; 43.0 [C-2']. **^{19}F NMR** (376 MHz, CD_3OD): δ 15.2 [s, reference: CF_3CD_2OD]. **ESI-MS** (positive ions): for $C_{10}H_{13}F_3N_3O_4$, calcd.: 296.09; found 296.02 ($M+H^+$); for $C_{10}H_{12}F_3N_3O_4Na$, calcd.: 318.07; found 318.47 ($M+Na^+$).

Synthesis of 2 on a large scale. To a solution of 2'-deoxycytidine (0.50 g, 2.20 mmol, 1 eq) in 3 ml of H_2O, CF_3SO_2Na (1.03 g, 6.60 mmol, 3 eq) dissolved in H_2O (5 ml) was added, followed by a 70% aq. solution of *tert*-BuOOH (1.05 ml, 11.0 mmol, 5 eq) which was added dropwise to the mixture at 0 °C. Then the reaction was then taken to r.t. After 6 h, the reaction was quenched by addition of a satd. aq. solution of $NaHCO_3$ (3 ml) until neutralization, and the resulting mixture taken to dryness. The crude was then resuspended in CH_3OH, aided by sonication, and filtered off to eliminate the $NaHCO_3$ solid precipitate. The filtered solution was purified on a silica gel column eluted with a gradient from 5 to 15 % of CH_3OH in CH_2Cl_2, giving 440 mg of the target 5-CF_3-2'-deoxycytidine **2** (1.49 mmol, 68 % yield) and 127 mg (26 %) of the starting nucleoside.

Trifluoromethylation of 2'-deoxyadenosine.

Synthesis of 3',5'-*O*-diacetyl-2'-deoxyadenosine. 2'-deoxyadenosine (**5a**, 200 mg, 0.80 mmol, 1.0 eq) was coevaporated twice and then suspended in anhydrous acetonitrile (5.0 ml). To the white suspension, DMAP (7.8 mg, 0.064 mmol, 0.08 eq), TEA (293 µl, 2.10 mmol, 2.6 eq) and acetic anhydride (180 µl, 1.91 mmol, 2.4 eq) were added, under stirring, in the order. After 3 h, the reaction was quenched by addition of methanol (3 ml) and taken to dryness under reduced pressure. The crude was partitioned between CH_2Cl_2 (10 mL) and water (10 mL), the organic phase was separated and the aqueous layer was back-extracted five times with CH_2Cl_2 (5 x 10 mL). The combined organic phases were then taken to dryness and redissolved in few drops of CH_2Cl_2. Addition to the mixture of *n*-hexane/Et_2O 1:1 (v/v) led to the formation of a white precipitate, which afforded 242 mg (0.72 mmol, 90 % yield) of desired product **6a**. The identity and purity of the isolated product were confirmed by ^{1}H and ^{13}C-NMR spectra and by ESI-MS spectra (data not shown).

Trifluoromethylation of 3',5'-*O*-diacetyl-2'-deoxyadenosine. To a solution of **6a** (90 mg, 0.27 mmol, 1.0 eq) in CH_2Cl_2 (5 ml), CF_3SO_2Na (63 mg, 0.40 mmol, 1.5 eq), previously dissolved in water (2.0 ml), and, subsequently, *tert*-ButOOH (70% aq. solution, 65 µL, 0.67 mmol, 2.5 equiv) were added dropwise at 0 °C under stirring. The reaction, monitored by TLC, was then taken at room temperature for 4 h. Further additions of the reactants CF_3SO_2Na (63 mg, 1.5 eq) and *tert*-BuOOH (65 µL, 2.5 equiv) were performed after additional 4, 24, 36 and 48 h. After 60 h the reaction mixture was partitioned between CH_2Cl_2 (20 mL) and a satd. aq. $NaHCO_3$ solution (20 mL). The organic layer was separated, and the aqueous phase was extracted with CH_2Cl_2 (3x 10 mL). The combined organic layers were concentrated and purified by column chromatography on silica gel. Elution with a gradient of AcOEt in CH_2Cl_2 (from 30 to 40%) allowed the isolation of target compound **7a** (21 mg, 0.051 mmol, 19 % yield). In addition to the desired product, more apolar compounds could be isolated, identified by ^1H and ^{13}C NMR (data not shown) as the compounds derived by *N*-glycosidic bond cleavage in the trifluoromethylated nucleoside. Finally, elution of the column with CH_2Cl_2/CH_3OH 9:1 (v/v) afforded 30 mg (0.090 mmol, 33%) of the starting nucleoside **6a**. Thus trifluoromethylated nucleoside **7a**, taking into account the recyclable starting material, was obtained with a recovery yield of 28 %.

7a: R_f = 0.4 in 1:1 CH_2Cl_2/AcOEt (v/v). **^{1}H NMR** (200 MHz, $CDCl_3$): δ 8.40 [s, 1H, H-2]; 6.39 [dd, *J* = 7.6 and 6.4 Hz, 1H, H-1']; 5.93 [bs, 2H, NH_2-6]; 5.62 [m, 1H, H-3']; 4.60 – 4.28 [overlapped signals, 3H, H-4' and H_2-5']; 3.74 [m, 1H, H-2'$_a$]; 2.44 [m, 1H, H-2'$_b$]; 2.14 and 2.06 (s's, 3H each**,** 2x C*H*$_3$CO). **^{13}C NMR** (50 MHz, CD_3OD): δ 170.5 and 170.2 [2x CH_3*C*O]; 156.3 [C-6]; 154.5 [C-2]; 150.9 [C-4]; 141.7 [q, *J* = 41 Hz, C-8]; 118.5 [C-5]; 118.3 [q, *J* = 270 Hz, CF_3]; 85.3 [C-1']; 82.9 [C-4']; 74.5 [C-3']; 63.4 [C-5']; 35.1 [C-2']; 20.9 and 20.7 [2x *C*H$_3$CO]. **^{19}F NMR** (376 MHz, CD_3OD): δ 15.9 [s, reference: CF_3CD_2OD]. **ESI-MS** (positive ions): for $C_{15}H_{16}F_3N_5O_5Na$, calcd: 426.10; found 426.60 ($M+Na^+$).

Synthesis of 8-CF_3-2'-deoxyadenosine. Compound **7a** (20 mg, 0.049 mmol) was dissolved in 2.0 ml of 7 N methanolic ammonia at 0 °C and the reaction was kept at 4 °C for 20 h. Then, the crude was taken to dryness and purified by column chromatography. Elution of the silica gel column with increasing amounts of CH_3OH in CH_2Cl_2 (from 1 to 5 %) afforded 16 mg of compound **8a** (0.048 mmol), pure from TLC and NMR control, in 98% yield.

8a: R_f = 0.4 in 9:1 CH_2Cl_2/CH_3OH (v/v). **^{1}H NMR** (500 MHz, CD_3OD): δ 8.24 [s, 1H, H-2]; 6.45 [dd, *J* = 6.0 and 6.0 Hz, 1H, H-1']; 4.64 [d, *J* = 5.5 Hz, 1H, H-3']; 4.13 [bs, 1H, H-4']; 3.92 – 3.75

[AB part of an ABX system, J = 12.5 and 3.0 Hz, 2H, H_2-5']; 3.10 [m, 1H, H-2'$_a$]; 2.31 [dd, J = 13.0 and 5.5 Hz, 1H, H-2'$_b$]. **^{13}C NMR** (125 MHz, CD_3OD): δ 159.5 [C-6]; 155.8 [C-2]; 151.7 [C-4]; 139.1 [q, J = 39 Hz, C-8]; 120.6 [q, J = 269 Hz, CF_3]; 120.4 [C-5]; 91.4 [C-1']; 88.9 [C-4']; 74.1 [C-3']; 64.8 [C-5']; 41.4 [C-2']. **^{19}F NMR** (376 MHz, CD_3OD): δ 15.6 [s, reference: CF_3CD_2OD]. **ESI-MS** (positive ions): for $C_{11}H_{12}F_3N_5O_3Na$, calcd: 342.08; found 342.98 ($M+Na^+$).

Trifluoromethylation of 2'-deoxyguanosine.

Synthesis of 3',5'-*O*-diacetyl-2'-deoxyguanosine. To a suspension of 2'-deoxyguanosine (400 mg, 1.40 mmol) in anhydrous CH_3CN (10 mL), DMAP (14 mg, 0.12 mmol), TEA (514 µL, 3.7 mmol) and acetic anhydride (1.65 ml, 3.34 mmol) were sequentially added at rt under stirring. After 2 h, the reaction was quenched by addition of methanol and the solvent completely evaporated. The residue was resuspended in diethyl ether aided by sonication. The obtained white precipitate was collected by filtration and washed with several volumes of Et_2O/ethanol 9:1 (v/v) and successively dried under reduced pressure, affording 467 mg (1.33 mmol, 95% yield) of the target derivative **6b**. The identity and purity of the isolated product were confirmed by ^{1}H and ^{13}C-NMR spectra and by ESI-MS spectra (data not shown).

Trifluoromethylation of 3',5'-*O*-diacetyl-2'-deoxyguanosine.

To a suspension of nucleoside **6b** (67 mg, 0.19 mmol) in CH_2Cl_2 (5.0 mL) and H_2O (3.0 mL), CF_3SO_2Na (89 mg, 0.57 mmol, 3 eq) dissolved in H_2O (1.0 mL) was added under stirring. Then a solution of *tert*-ButOOH (70% aq. solution, 90 µl, 0.95 mmol, 5 eq) was added dropwise at 0 °C under stirring. After 24 h a further aliquot of CF_3SO_2Na (3 eq) and *tert*-ButOOH (5 eq) was added to the reaction mixture. After 48 h, the reaction was quenched by addition of a satd. aq. $NaHCO_3$ solution until neutralization and the biphasic system was transferred into a separatory funnel and diluted with CH_2Cl_2 and H_2O. The organic phase was separated and the aqueous layer was back-extracted five times with CH_2Cl_2 (5 x 10 mL). The combined organic phases were then taken to dryness and the crude was purified by silica gel chromatography, eluting the column with a gradient of CH_3OH in CH_2Cl_2 (from 0 to 10%), affording 31 mg (0.074 mmol, 39% yield) of target derivative **7b**.

7b: R_f = 0.40 in 9:1 (CH_2Cl_2/CH_3OH, v/v). **^{1}H NMR**: (500 MHz, $CDCl_3$): δ 6.87 [bs, NH_2-2]; 6.25 [dd, J = 6.5 and 7.5 Hz, 1H, H-1']; 5.51 [m, 1H, H-3']; 4.70 [m, 1H, H-5'$_a$]; 4.40 [m, 1H, H-5'$_b$]; 4.34 [m, 1H, H-4']; 3.47 [m, 1H, H-2'$_a$]; 2.41 [m, 1H, H-2'$_b$]; 2.14 and 2.01 [s's, 3H each, 2x CH_3CO]. **^{13}C NMR** (125 MHz, $CDCl_3$): δ 171.1 [CH_3CO]; 170.2 [CH_3CO]; 159.1 [C-6]; 154.3 [C-2]; 152.6 [C-4]; 134.6 [q, *J* = 41 Hz, C-8]; 118.5 [q, *J* = 269 Hz, CF_3]; 116.6 [C-5]; 85.5 [C-1']; 82.8 [C-4']; 74.6 [C-3']; 63.4 [C-5']; 35.1 [C-2']; 21.0 and 20.8 [2x CH_3CO]. **^{19}F NMR** (376 MHz, $CDCl_3$): δ 16.1 [s, reference: CF_3CD_2OD]. **ESI-MS** (positive ions): for $C_{15}H_{16}F_3N_5O_6$, calcd. 419.11; found 420.85 ($M+H^+$).

Synthesis of 8-CF_3-2'-deoxyguanosine.

Compound **7b** (25 mg, 0.059 mmol) was dissolved in 2.0 ml of 7 N methanolic ammonia at 0 °C and the reaction was kept at 4 °C for 24 h. Then, the solvent was evaporated and the crude was purified by silica gel column chromatography, eluting the column with increasing amounts of CH_3OH (from 2 to 10 %) in CH_2Cl_2. Target nucleoside **8b** was obtained in an almost quantitative yield (20 mg, 0.059 mmol), pure from TLC and NMR control.

8b: R_f = 0.45 in 85:15 (CH_2Cl_2/CH_3OH, v/v). **^{1}H NMR**: (500 MHz, CD_3OD): δ 6.30 [dd, *J* = 7.5 and 7.5 Hz, 1H, H-1']; 4.61 [m, 1H, H-3']; 4.05 [dd, *J* = 3.5 and 6.0 Hz, 1H, H-4']; 3.90 - 3.65 [AB part of an ABX system, *J* = 12.5 and 3.5 Hz, 2H, H_2-5']; 3.15 [m, 1H, H-2'$_a$]; 2.25 [A part of an ABX system, *J* = 2.5 and 6.0 Hz, 1H, H-2'$_b$]. **^{13}C NMR** (125 MHz, CD_3OD): δ 160.0 [C-6]; 157.3 [C-2]; 156.2 [C-4]; 136.5 [q, *J* = 41 Hz, C-8]; 121.2 [q, *J* = 269 Hz, CF_3]; 118.5 [C-5]; 90.5 [C-1']; 87.9 [C-4']; 73.7 [C-3']; 64.4 [C-5']; 40.3 [C-2']. **^{19}F NMR** (376 MHz, CD_3OD): δ 15.0 [s, reference: CF_3CD_2OD]. **ESI-MS** (positive ions): for $C_{11}H_{12}F_3N_5O_4Na$, calcd: 358.07; found 358.67 ($M+Na^+$).

Trifluoromethylation of inosine.

Synthesis of 8-CF_3-inosine.

To a solution of inosine (50 mg, 0.19 mmol, 1 eq) in 1.5 ml of H_2O, CF_3SO_2Na (87 mg, 0.56 mmol, 3 eq) previously dissolved in H_2O (1 ml) and, successively, a 70% aq. solution of *tert*-BuOOH (90 µl, 0.95 mmol, 5 eq) were added at 0 °C. The reaction was then taken to r.t. and, after 6 h, a second portion of CF_3SO_2Na (87 mg in 1 ml H_2O) and *tert*-BuOOH (90 µl) was added to the

reaction mixture at 0 °C. TLC control after 24 h revealed the formation of a new product, with higher R_f than the starting material. The reaction was quenched by addition of a satd. aq. solution of $NaHCO_3$ (3 ml) and the resulting mixture taken to dryness. The crude was resuspended in CH_3OH, aided by sonication, and filtered off to remove the precipitate. The filtered solution was purified on a silica gel column eluted with a gradient from 5 to 15 % of CH_3OH in CH_2Cl_2, giving 46 mg of the target compound **9** (0.16 mmol, 73 % yield).

9: R_f = 0.*5* in 8:2 CH_2Cl_2/CH_3OH (v/v). **^{1}H NMR** (500 MHz, D_2O): δ 8.33 [s, 1H, H-2]; 6.13 [d, *J* = 6.0 Hz, 1H, H-1']; 5.12 [apparent t, *J* = 6.0 and 6.0 Hz, 1H, H-2']; 4.56 [dd, *J* = 3.0 and 6.0 Hz, 1H, H-3']; 4.32 [dd, *J* = 3.0 and 4.0 Hz, 1H, H-4']; 3.98 – 3.90 [AB part of an ABX system, *J* = 12.5 and 4.0 Hz, 2H, H_2-5']. **^{13}C NMR** (125 MHz, D_2O): *δ* 160.9 [C-6]; 151.6 [C-4]; 147.5 [C-2]; 140.8 [C-8]; 125.1 [C-5]; 124.5 [q, J = 270 Hz, CF_3]; 90.6 [C-1']; 87.4 [C-4']; 74.1 [C-2']; 71.3 [C-3']; 64.3 [C-5']. **^{19}F NMR** (376 MHz, CD_3OD): δ 15.9 [s, reference: CF_3CD_2OD]. **ESI-MS** (positive ions): for $C_{11}H_{11}F_3N_4O_5Na$, calcd: 359.06; found 360.01 ($M+Na^+$).

Cell cultures and microcultures bioassay.

The biological activity of the trifluoromethylated nucleosides was investigated on tumour MCF-7 human breast adenocarcinoma cells, DU-145 human prostate cancer cells and C6 rat glioma cell line, and on non tumour HaCaT human skin keratinocytes and L6 rat myoblasts, all purchased from ATCC® (American Type Culture Collection, Manassas, Virginia, USA). DU-145, C6, HaCaT and L6 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) containing high glucose (4.5 g/l), while MCF-7 cells were grown in RPMI 1640 medium (Invitrogen, Paisley, UK). Media were supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/ml, Sigma) and streptomycin (100 µg/ml, Sigma), according to ATCC recommendations. All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C. For bioactivity and cytotoxicity studies, cells were seeded in a 96-microwell culture plates at density of 10^4 cells/well. Cells were allowed to grow for 24 h, then the medium was replaced with fresh medium and cells were treated for further 48 h with different concentrations of the tested compounds. In detail, compounds **2**, **8a** and **8b** were dissolved in H_2O and 1 or 2 µL of aqueous solutions were added to cell culture medium to give various concentration ranging from 1 to 250 µM. Positive control for cytotoxicity was performed using cisplatin (purchased from Sigma). Live/dead cell number was determined by the trypan blue dye exclusion test. Briefly, after the treatments, the medium was removed and the cells were washed

twice with PBS buffer solution (Sigma) and then incubated with a trypsine-EDTA solution (Sigma) at 37°C for 5 min. Trypsine was inactivated by re-suspending the cells in medium containing 10% FBS (Cambrex). The cells were pelleted at 250 x g and resuspended in PBS. Viable cells, cells that excluded 0.4% trypan blue (Sigma), were then counted with a Burker haemocytometer chamber. Concurrently, cell viability was evaluated with an MTT assay procedure, which measures the level of mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as substrate. The assay was based on the redox ability of living mitochondria to convert dissolved MTT into insoluble formazan. In brief, after treatments with the tested compounds, the medium was removed and the cells were incubated with 20 µL/well of an MTT solution (5 mg/mL) for 1 h in a humidified 5% CO_2 incubator at 37°C. The incubation was stopped by removing the MTT solution and by adding 100 µL/well of DMSO to solubilize the formazan. Finally, the absorbance was monitored at 550 nm by using a Perkin-Elmer LS 55 Luminescence Spectrometer (Perkin-Elmer Ltd, Beaconsfield, UK). The antiproliferative activity of the investigated nucleosides was reported by a "cell survival index", arising from the combination of cell viability evaluation with cell counting. The calculation of the concentration required to inhibit the net increase in the 48 h cell count and viability by 50% (IC_{50}) is based on plots of data carried out in triplicates and repeated three times. The IC_{50} values against various different tumour cell lines were investigated using standard *in vitro* antiproliferation assays and were obtained using a dose response curve by nonlinear regression using a curve fitting program, GraphPad Prism 5.0, and are expressed as mean ± SEM.

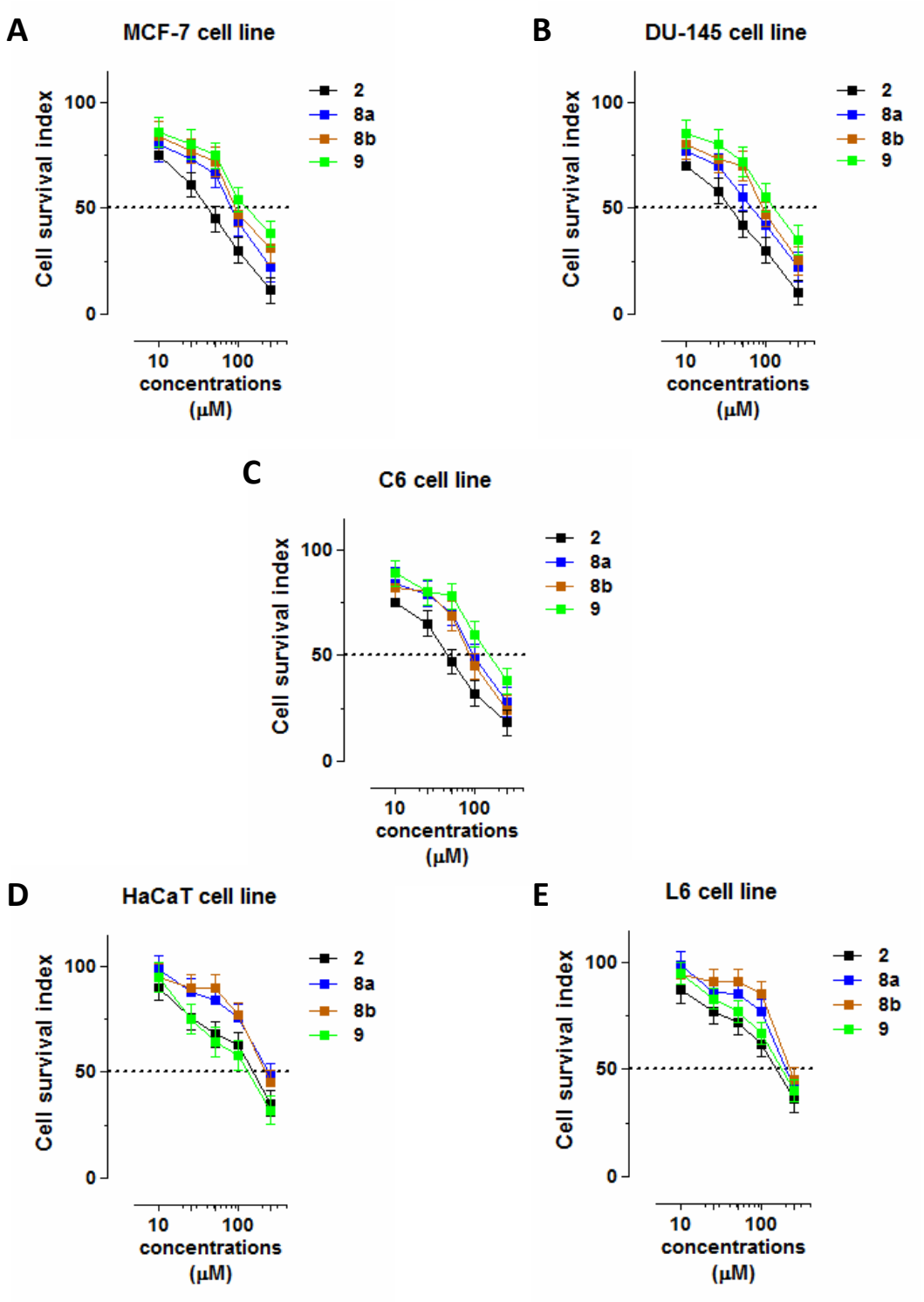

FIGURE S1. Cell survival index values, evaluated by MTT assay and total cell count, in MCF-7 (panel A), DU-145 (panel B), C6 (panel C), HaCaT (panel D) and L6 (panel E) cell lines incubated for 48 h with different concentrations of compounds **2**, **8a**, **8b** and **9**, as indicated in the legends. Data are expressed as percentage of untreated control cells and are reported as mean of three independent experiments ± SEM (n=3).