SUPPORTING INFORMATION

Nitric oxide donor doxorubicins accumulate into doxorubicin resistant human colon cancer cells inducing cytotoxicity

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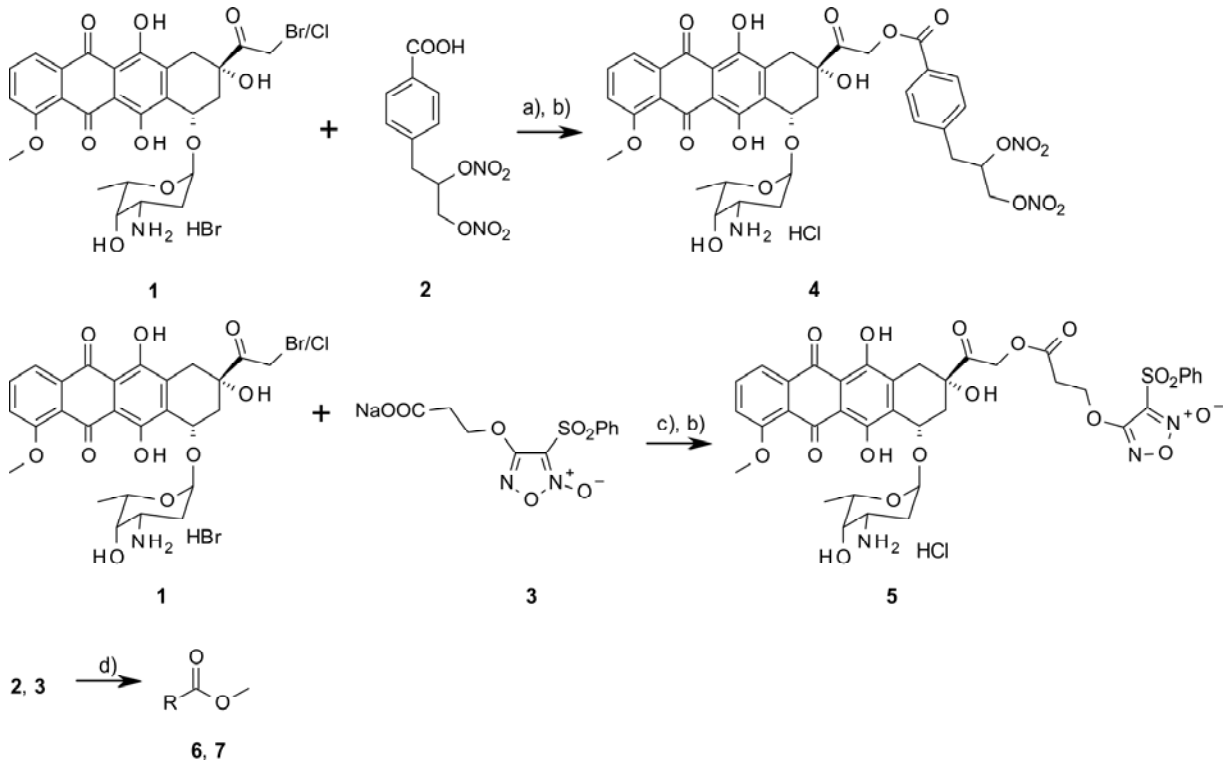
Supplementary Experimental Section

Chemistry. ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 at 300 and 75 MHz respectively, using $SiMe_4$ as the internal standard. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. ESI MS spectra were recorded with Agilent LC/MSD TRAP SL. Melting points were determined with a capillary apparatus (Büchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM); PE stands for 40-60 petroleum ether. The progress of the reactions was followed by thin layer chromatography (TLC) on 5×20 cm plates with a layer thickness of 0.2 mm. Anhydrous magnesium sulfate was used as the drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Purities of all new compounds ≥ 90% were determined by HPLC. **1**,[1] and **2**[2] were obtained as described elsewhere.

The purity of **4** and **5** was assessed by RP-HPLC. Analyses were performed on a HP1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA). The analytical column was a Nucleosil 100-5C18 Nautilus (250×4.6 mm, 5 mm particle size) (Macherey-Nagel). Compounds were dissolved in CH_3CN/water and injected through a 20 μL loop. The mobile phase consisted of 0.1% aqueous HCl (solvent A) and CH_3CN (solvent B) and elution was in gradient mode (initially 35% of solvent B, which increased linearly to 65% over 10 min). HPLC retention times (t_R) were obtained at flow rates of 1.0 mL min^{-1}, and the column effluent was monitored at 233, 476 and 495 nm referenced against 700 nm. Compound **4**: t_R = 11.2 min, P_{HPLC} = 98.1%; compound **5**: t_R = 9.7 min, P_{HPLC} = 93.0%.

Scheme 1.[a]



[a]Conditions: (a) K_2CO_3, acetone, r.t.; (b) THF dry, HCl in dioxane dry; (c) acetone, reflux; (d) H_2SO_4, MeOH, reflux.

3-[(3-Phenylsulfonyl)furoxan-4-yloxy]propanoic acid (**3**). A solution of Jones reagent 2.5 M (19 mL, 46.62 mmol) was added to a stirred solution of 3-[(3-phenylsulfonyl)furoxan-4-yloxy]propan-1-ol[3] (5.6 g, 18.65 mmol) in acetone (150 mL), cooled at 0 °C. The mixture was allowed to reach r.t. and stirred for 4h. iPrOH (10 mL) was added and the mixture was concentrated under reduced pressure. The residue was dissolved EtOAc (150 mL) and was extracted with a saturated solution of $NaHCO_3$ (3 × 20 mL). The aqueous layers were acidified with 6M HCl and extracted twice with EtOAc (50 mL). The combined organic layers were dried, filtered and concentrated under reduced pressure to give the title compound as white solid; yield 65 %; m.p. 142-143 °C (toluene); ^{1}H-NMR (DMSO-d_6) δ 2.81 (t, 2H), 4.59 (t, 2H), 7.74 (t, 2H), 7.91 (t, 1H), 8.00 (d, 2H), 12.63 (sbr, 1H); ^{13}C-NMR (DMSO-d_6) δ 33.3, 67.4, 110.5, 128.4, 130.1, 136.3, 137.3, 158.8, 171.5. MS (CI) *m/z* 315 $(M+1)^+$. *Anal*. Calcd. for $C_{11}H_{10}N_2O_7S$: C, 42.04; H, 3.21; N, 8.91. Found: C, 42.51; H, 3.27; N, 8.97.

Adriamycin 14-O-[4-(2,3-dinitrooxypropyl)benzoate] hydrochloride (4). To a solution of **2** (0.32 g, 1.12 mmol) in acetone (25 mL) **1** (0.50 g, 0.75 mmol) and K_2CO_3 (0.26 g, 1.90 mmol) were added at r.t. Reaction mixture was vigorously stirred at r.t. for 4h, then it was filtered through Celite pad, the precipitate was washed with acetone and the filtrate was evaporated. The obtained solid residue was purified by flash chromatography (eluent: gradient from 95 / 5 to 8 / 2 CH_2Cl_2 / MeOH v / v) to give a red powder. The obtained product was dissolved in dry THF and 2.36 M HCl solution in dry dioxane (0.25 mL, 0.59 mmol) was added. Obtained precipitate was filtered, washed with Et_2O and dessicated; yield 30 %; ^{1}H-NMR (CD_3OD): 1.37 (d, 3H), 1.94-2.12 (m, 3H), 2.52 (d, 1H), 2.75 (d, 1H), 3.06 (d, 1H), 3.21 (m, 2H), 3.64 (m, 1H), 3.76 (s, 1H), 3.95 (s, 3H), 4.37 (q, 1H), 4.63 (dd, 1H), 4.95 (dd, 1H), 5.34-5.61 (4H, m), 5.70 (q, 1H), 7.42 (m, 1H), 7.49 (d, 2H), 7.70 (m, 2H), 8.07 (d, 2H); ^{13}C-NMR (CD_3OD): 17.1, 29.5, 33.4, 36.2, 37.0, 57.1, 67.9, 68.1, 71.4, 72.4, 77.4, 81.0, 101.2, 112.0, 112.2, 120.4, 120.5, 121.1, 130.0, 130.9, 131.3, 135.1, 135.4, 136.1, 137.2, 142.6, 156.0, 157.2, 162.3, 167.2, 187.5, 187.5, 208.7; MS (ESI) *m/z* 812 $(M+1)^+$.

Adriamycin 14-O-{[(3-phenylsulfonyl)-4-yloxy]propanoate} hydrochloride (5). To a solution of $NaHCO_3$ (0.40 g, 4.76 mmol) in H_2O (70 mL) **3** (1.40 g, 4.46 mmol) was added and the reaction mixture was stirred until became clear. Obtained solution was filtered and lyophilized to give a sodium salt of **3** as a white powder. Obtained solid was suspended in acetone (70 mL) and **1** (0.60 g, 0.9 mmol) was added in one portion. Reaction mixture was refluxed for 12h, then filtered through Celite pad, the precipitate was washed with acetone and the filtrate was evaporated. The obtained solid residue was purified by flash chromatography (eluent gradient from 9 / 1 to 8 / 2 CH_2Cl_2 / MeOH v / v) to give a red solid. The obtained product was suspended in dry THF and 2.36 M HCl solution in dry dioxane (0.2 mL, 0.47 mmol) was added at -10 °C. Suspension was vigorously stirred at -10 °C for 2h, then precipitate was filtered, washed with Et_2O and dessicated; yield 10 %; ^{1}H-NMR (DMSO-d_6): 1.11 (d, 3H), 1.66 (d, 1H), 1.83 (t, 1H), 2.01 (m, 1H), 2.27 (d, 1H), 2.73 (d, 1H), 2.99-3.06 (m, 2H), 3.56 (s, 1H), 3.91 (3H, s), 4.22 (q, 1H), 4.60 (m, 2H), 4.85 (m, 1H), 5.23-5.30 (m, 3H), 5.44 (m, 1H), 5.79 (s, 1H), 7.55-7.95 (m, 11H), 13.14 (s, 1H), 13.95 (s, 1H); MS (ESI) *m/z* 840 $(M+1)^+$.

Methyl 4-(2,3-dinitroxypropyl)benzoate (6). To a solution of **2** (0.43 g, 1.50 mmol) in methanol (25 mL) 2 drops of conc. sulfuric acid were added and reaction mixture was refluxed for 12 h. Then it was poured in H_2O (100 mL) and extracted twice with Et_2O (30 mL). The organic layer was washed with $NaHCO_3$ sat. sol., then with brine, dried and organic solvent was evaporated. Obtained oil was purified by flash chromatography (eluent PE / CH_2Cl_2 8 / 2 v / v) to give the title compound as a colorless oil that became solid on standing; yield 80%; m.p. 47.0 – 47.5 °C (C_6H_{14}); ^{1}H-NMR ($CDCl_3$): 3.04 – 3.19 (m, 2H), 3.92 (s, 3H), 4.44 (dd, 1H), 4.75 (dd, 1H), 5.44 – 5.52 (m, 1H), 7.33 (d, 2H), 8.03 (d, 2H); ^{13}C-NMR ($CDCl_3$): 35.6, 52.2, 70.1, 78.9, 129.6, 130.0, 130.6, 139.3, 166.6; MS (CI) *m/z* 301 $(M+1)^+$. *Anal*. Calcd. for $C_{11}H_{12}N_2O_8$: C, 44.01; H, 4.03; N, 9.33. Found: C, 44.18; H, 4.17; N, 9.08.

Methyl 3-[(3-phenylsulfonyl)-4-yloxy]propanoate (**7**). To a solution of **3** (0.42 g, 1.34 mmol) in dry methanol (15 mL) 2 drops of conc. sulfuric acid were added and reaction mixture was refluxed for 2 h. Then it was cooled in ice bath and the precipitate formed was filtered, washed with small amount of cold methanol and crystallized from methanol, to give the title compound as white crystalline solid; yield 55%; m.p. 108 – 108.5 °C (MeOH); ^{1}H-NMR ($CDCl_3$): 2.92 (t, 2H), 3.77 (s, 3H), 4.70 (t, 2H), 7.62 (t, 2H), 7.76 (t, 1H), 8.04 (d, 2H); ^{13}C-NMR ($CDCl_3$): 33.4, 52.2, 66.6, 111.3, 128.6, 129.7, 135.6, 138.1, 158.6, 170.1; MS (CI) *m/z* 329 $(M+1)^+$. *Anal*. Calcd. for $C_{12}H_{12}N_2O_7S$: C, 43.90; H, 3.68; N, 8.53. Found: C, 43.71; H, 3.75; N, 8.55.

Biological assays

Chemicals. Fetal bovine serum (FBS), penicillin-streptomycin (PS) and RPMI 1640 medium were supplied by Sigma Chemical Co (St.Louis, MO), plasticware for cell culture was from Falcon (BD Biosciences, Bedford, MA). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA), the protein content of cell monolayers and cell lysates was assessed with the bicinchoninic acid kit from Sigma Chemical Co. Doxorubicin and all the other reagents were purchased from Sigma Chemical Co.

Cells culture. HT29-dx, a doxorubicin resistant human colon cancer cell line was created from parental doxorubicin-sensitive HT29 cells, as previously reported.[4] Cells were cultured in RPMI 1640 medium containing 150 nM doxorubicin, 10 % v / v FBS and 1 % v / v PS, and maintained in a humidified atmosphere at 37 °C and 5 % CO_2. HT29-dx cells exhibited a higher amount of P-gp and MRP3, had a lower intracellular accumulation of doxorubicin and were more resistant to the drug toxic effects if compared to HT29 cells.[4]

Drug accumulation and efflux. Cells were grown in 60 mm diameter Petri dishes. Before every test, cells were washed and cultured in fresh medium without doxorubicin for 24 hours. Cells were incubated in RPMI 1640 medium containing doxorubicin, **4**, or **5** (in the experimental conditions indicated under Results), washed twice in ice-cold PBS, detached with trypsin/EDTA (0.05 / 0.02 % v / v), centrifuged for 30 seconds at 13,000 × g and re-suspended in 1 mL of a 1 / 1 mixture of ethanol / 0.3 N HCl. 50 μL of cell suspension were sonicated on crushed ice with two 10 seconds bursts (Labsonic sonicator, 100 W) and used for measurement of cellular proteins; the remaining part was checked for the drug content using a Perkin-Elmer LS-5 spectrofluorimeter (Perkin-Elmer, Waltham, MA). Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was prepared in the absence of cells in every set of experiments and its fluorescence was subtracted from that obtained in the presence of cells. Fluorescence was converted in nmoles of drug per mg of cellular protein, using a calibration curve prepared previously.

In order to calculate the kinetics of the drug efflux, we incubated the cell monolayers with 1 to 200 μM doxorubicin, **4**, or **5** for 10 min; cells were then washed with PBS, re-suspended in 1 mL of ethanol / 0.3 N HCl and analyzed for the intracellular drug content as described above. In parallel, other dishes, after incubation under the same experimental conditions, were washed with PBS, left for a further 10 min in PBS at 37 °C, then washed again, re-suspended and tested for intracellular drug content. The difference between the two types of cellular samples under each experimental condition was expressed as nmoles of drug extruded per min per mg of cellular protein. The doxorubicin efflux per unit of time

(dc / dt) was plotted versus the intracellular drug concentration at time 0. V_{max} of the drug efflux was estimated using the Enzfitter software (Biosoft Corporation, Cambridge, United Kingdom).

Cytotoxicity assay. The activity of the lactate dehydrogenase (LDH) in the extracellular medium was measured, as a sensitive index of the drugs cytotoxic effects.[4] The medium was centrifuged at 12,000 × *g* for 15 min to pellet cellular debris, whereas cells were washed with fresh medium, detached with trypsin / EDTA, re-suspended in 0.2 mL of 82.3 mM triethanolamine phosphate-HCl (pH 7.6) and sonicated on ice with two 10 seconds bursts. LDH activity was measured in the extracellular medium and in the cell lysate: 50 μL of supernatant from extracellular medium or 5 μL of cell lysate were incubated at 37 °C with 5 mM NADH. The reaction was started by adding 20 mM pyruvic acid and was followed for 6 min, measuring absorbance at 340 nm with Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The reaction kinetics was linear throughout the time of measurement. Both intracellular and extracellular enzyme activity was expressed in μmol NADH oxidized / min / dish, then extracellular LDH activity was calculated as percentage of the total LDH activity in the dish.

Nitrite production. Confluent monolayers in 35 mm diameter Petri dishes were incubated in fresh medium under the experimental conditions indicated in Results. Nitrite production was measured by adding 0.15 mL of cell culture medium to 0.15 mL of Griess reagent in a 96-well plate, and after a 10 min incubation at 37 °C in the dark, the absorbance was detected at 540 nm with a Packard EL340 microplate reader (Bio-Tek Instruments). A blank was prepared for each experiment in the absence of cells, and its absorbance was subtracted from that obtained in the presence of cells. Nitrite concentration was expressed as nanomoles of nitrite / mg of cell protein.

Western blot analysis. Cells grown at confluence on 60 mm diameter Petri dishes were washed twice with PBS, then lysed in sample buffer heated at 99 °C (25 mM Hepes, 135 mM NaCl, 1% v/v Nonidet P-40, 5 mM EDTA, 1 mM EGTA, 1 mM $ZnCl_2$ and 10 % v / v glycerol) and sonicated with one 10

seconds burst. After centrifugation (13,000 × *g* for 15 min), the protease inhibitor cocktail set III (100 mmol / L AEBSF, 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin and 1 mM pepstatin; Calbiochem, La Jolla, CA), 2 mM phenylmethylsulfonyl fluoride and 1 mM $NaVO_4$ were added to the supernatant. Whole cell extracts containing equal amounts of proteins (30 μg) were separated by SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore, Bedford, MA) and probed with the following antibodies, diluted in 1 % w / v PBS-BSA: anti-Pgp (rabbit polyclonal, diluted 1 to 250, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-MRP3 (goat polyclonal, diluted 1 to 250, Santa Cruz Biotechnology Inc.); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; rabbit polyclonal, diluted 1 to 500, Santa Cruz Biotechnology Inc.). After an overnight incubation, the membrane was washed with PBS-Tween 0.1 % v / v and subjected for 1 h to a horseradish-conjugated anti-rabbit antibody (Bio-Rad, diluted 1 to 3000 in PBS-Tween with 5 % w / v non-fat dry milk) or to an anti-goat antibody (Santa Cruz Biotechnology Inc., diluted 1 to 1000 in PBS-Tween with non-fat dry milk 5%). The membrane was washed again with PBS-Tween and proteins were detected by enhanced chemiluminescence (Perkin-Elmer). To analyze the presence of nitrated proteins, the whole cell extract was subjected to an overnight immunoprecipitation using a rabbit polyclonal anti-nitrotyrosine antibody (diluted 1 to 100 in blocker non-fat dry milk 1%; Millipore). Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane sheets and probed with anti-Pgp and anti-MRP3 antibodies, as previously described.

Rhodamine assay. Cells were washed with fresh PBS, detached with Cell Dissociation Solution and re-suspended at 5 x 10^5 cells / mL in 1 mL RPMI medium containing 5% FBS. The samples were maintained at 37°C for 20 min in the presence of 1 μg / mL rhodamine 123. After this incubation time, cells were washed and re-suspended in 0.5 mL PBS, and the intracellular rhodamine content, which is inversely related to its efflux, was detected using a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ). For each analysis 100,000 events were collected and data were analyzed by the CellQuest software (Becton Dickinson).

Statistical Analysis.

All data in text and figures are provided as mean ± standard deviation (SD). The results were analysed by a one-way analysis of variance (ANOVA) and Tukey's test. $p < 0.05$ was considered significant.

Supplementary references

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(3) Cena, C.; Visentin, S.; Di Stilo, A.; Boschi, D.; Fruttero, R.; Gasco, A. Studies on agents with mixed NO-dependent and calcium channel antagonistic vasodilating activities. *Pharm. Res.* **2001**, *18*, 157-165.

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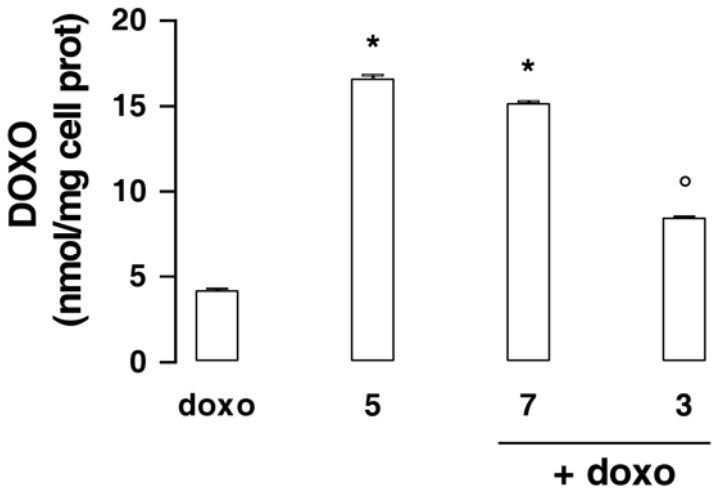

Figure 1. Intracellular accumulation of doxorubicin in cells pre-treated with phenylsulfonylfuroxan-derivatives.

HT29-dx cells were incubated for 24 h with 5 µmol / L doxorubicin (doxo), **5**, **7**, or **3**. In the last two experimental conditions cells were then washed and incubated with 5 µmol / L doxorubicin for additional 24 h. Then the intracellular doxorubicin content was measured fluorimetrically. Experiments were performed in duplicate (n = 3). Significance versus DOXO: * $p < 0.01$; versus 5: ° $p < 0.01$.

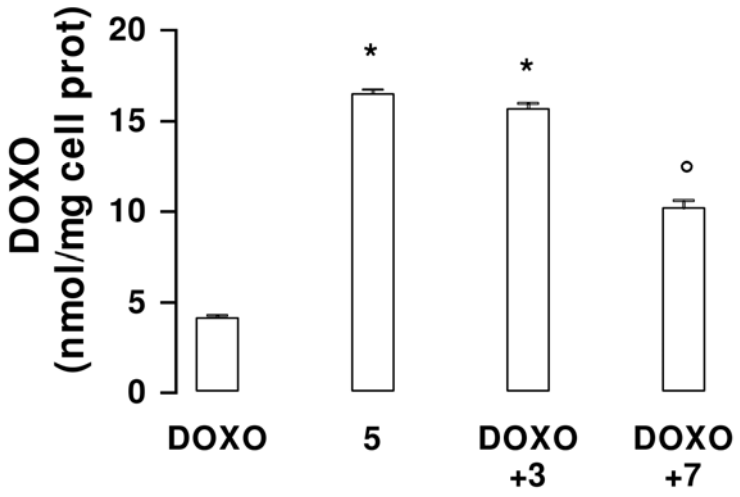

Figure 2. Intracellular accumulation of doxorubicin co-dosed with phenylsulfonylfuroxan-derivatives.

HT29-dx cells were incubated for 24 h with 5 µmol / L doxorubicin (doxo), **5**, or doxorubicin together with **7** or **3** at the same concentration. Then the intracellular doxorubicin content was measured fluorimetrically. Experiments were performed in duplicate (n = 3). Significance versus DOXO: * $p < 0.01$; versus **5**: ° $p < 0.05$.

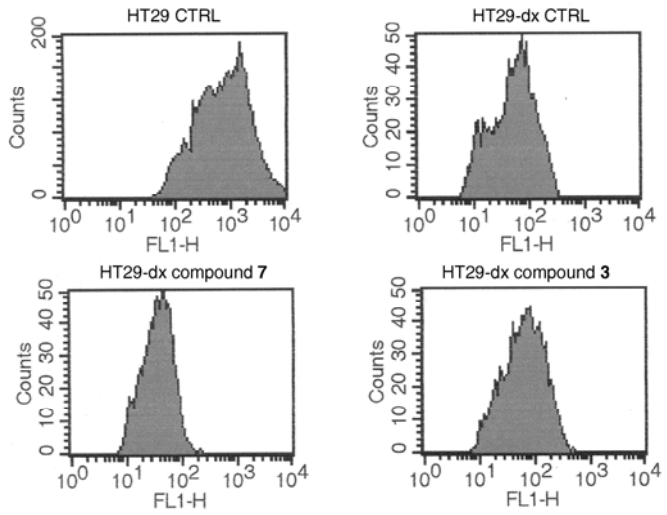

Figure 3. Rhodamine efflux in the presence of phenylsulfonylfuroxan-derivatives.

HT29-dx cells were grown 24 h with fresh medium (CTRL), 5 μmol / L **7**, or **3**, then washed and incubated for further 20 min with rhodamine. Untreated (CTRL) HT29 cells were used as control of a doxorubicin-sensitive cell line, with a low expression of P-gp. [4] The intracellular fluorescence of the P-gp substrate rhodamine, inversely related to its efflux, was assessed by flow cytometry analysis and is reported on x-axis (FL1-H; bandpass filter 530 nm); cells count is reported on y-axis. The figures shown here are representative of three similar experiments, performed in duplicate.