Doxorubicin-antioxidant multitarget drugs

Supplementary data for:

Doxorubicin-antioxidant co-drugs.

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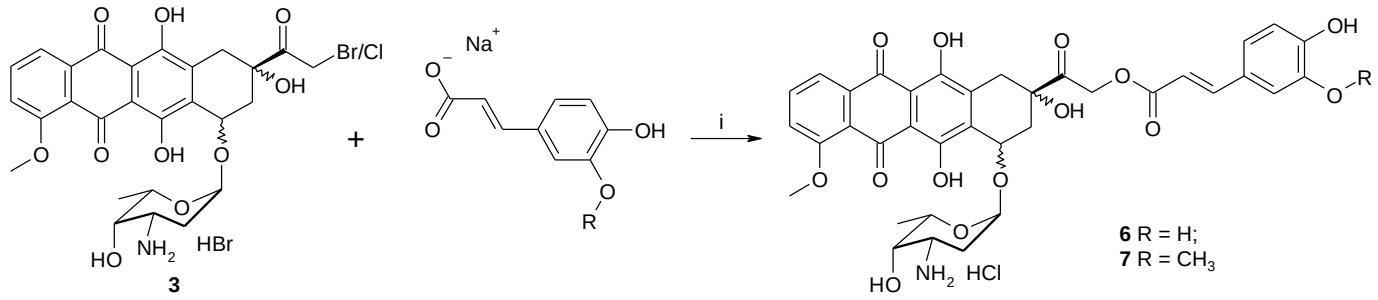
Chemistry

^{1}H NMR spectra were recorded on a Bruker Avance 300, at 300 MHz, using SiMe4 as internal standard; abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. ESI MS spectra were recorded on a Micromass Quattro API micro (Waters Corporation, Milford, MA, USA). Flash column chromatography was done on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM). The progress of reactions was monitored by thin layer chromatography (TLC) on 5×20 cm plates with a layer thickness 0.20 mm. Organic solvents were removed under vacuum at 30°C. Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone under a positive atmosphere of N_2. Compound **3** was synthesized by a published method.[1]

The purity of **6** and **7** was assessed by RP-HPLC and LC-MS. Analyses RP-HPLC were run with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), a diode-array detector (DAD) (model G1315B) integrated into the HP1100 system. Data were processed using a HP ChemStation system (Agilent Technologies). The analytical column was a Tracer Excel 120 ODS-B (250×4.6mm, 5 µm; Teknokroma, Barcelona). Compounds were dissolved in the mobile phase and injected through a 20 µL loop (Rheodyne, Cotati, CA). The mobile phase consisted of 0.1% aqueous HCOOH (solvent A) and CH_3CN (solvent B) and elution was in gradient mode: initially 35% of solvent B until 5 min, from 35 to 80% of solvent B between 5 and 10 min, 80% of solvent B until 20 min, and from 80 to 35% of solvent B between 20 and 25 min. HPLC retention times (t_R) were obtained at flow rates of 1.0 mL·min^{-1}, and the column effluent was monitored at 234 nm and 480 nm referenced against a 700 nm wavelength. Compound **6**: t_R = 9.6 min, PHPLC = 98.0%; compound 7: t_R = 10.1 min, PHPLC = 95.0%.

Analyses LC-MS were run with an Acquity UPLC system (Waters, Corporation, Milford, MA, USA) equipped with a diode-array detector (DAD) integrated into the UPLC system coupled via an electrospray ionization source to an Micromass Quattro API micro (Waters Corporation, Milford, MA, USA) mass spectrometer. Data were processed using a MassLynx system (Waters). The analytical column was a Phenomenex Synergy MAX-RP 80A (150×2.0 mm, 4 µm; Phenomenex Inc, Torrance, CA). Compounds were dissolved in the mobile phase and 5 µL were injected through an autosampler. The mobile phase consisted of 0.1% aqueous HCOOH (solvent A) and 0.1% HCOOH in CH_3CN (solvent B) and elution was in gradient mode: initially 30% of solvent B until 2 min, from 30 to 70% of solvent B between 2 and 4 min, 70% of solvent B until 6 min, and from 70 to 30% of solvent B between 6 and 7 min. UPLC retention times (t_R) were obtained at flow rates of 0.5 mL·min^{-1}, and the column effluent was monitored at 480 nm referenced against a 700 nm. The LCTQ capillary voltage was set to 3,5 kV and the cone voltage was set to +20 V. Full scan mass spectra were acquired by scanning between *m/z* 500 and 750 in the positive ion mode. Compound **6**: t_R = 1.85 min, purity = 97.0%; compound **7**: t_R = 3.09 min, purity = 95.0%.



i) Acetone, reflux.

Adriamycin 14-O-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoate] hydrochloride (6). Caffeic acid (0.54 g, 3.0 mmol) was dissolved in 40 mL of aqueous solution of $NaHCO_3$ (0.25 g, 2.0 mmol). The resulting solution was frozen in a dry ice–acetone bath and lyophilized to give a sodium salt as a white powder. The salt was suspended in acetone (100 mL) and daunorubicin 14-bromide/chloride (0.40 g, 0.60 mmol) was added. The resulting suspension was heated at reflux for 48 h, then filtered, and the solvent evaporated. The resulting solid was purified by flash chromatography (gradient from 95 / 5 to 8 / 2 CH_2Cl_2 / MeOH) to give a red solid, which was suspended in THF dry and 1.5 M HCl in dry dioxan (0.1 mL, 0.15 mmol) was added. The suspension was stirred for 4 h then filtered and the precipitate washed with Et_2O and desiccated. Yield 23 %; ^{1}H-NMR ($(CD_3)_2SO$): 1.18 (d, 3H), 1.70 (m, 1H), 1.89 (m, 1H) 2.08

(m, 1H), 2.32 (m, 1H); 2.88 (d, 1H), 3.09 (d, 1H), 3.62 (m, 1H), 3.98 (s, 3H), 4.27 (q, 1H), 4.94 (br. s, 1H), 5.29-5.41 (m, 3H), 5.78 (br. s, 1H), 6.36 (d, 1H), 6.80 (d, 1H), 7.03 (m, 1H), 7.11 (d, 1H), 7.54 (d, 1H), 7.64 (m, 1H), 7.89 (m, 2H); MS (ESI^+) *m/z* 706 $(M+1)^+$.

Adriamycin 14-O-[(2E)-3-(3-hydroxy-4-methoxyphenyl)prop-2-enoate] hydrochloride (7). Ferulic acid (0.75 g, 3.9 mmol) was dissolved in 100 mL of aqueous solution of $NaHCO_3$ (0.33 g, 3.9 mmol). The resulting solution was frozen in a dry ice–acetone bath and lyophilized to give a sodium salt as a white powder. This salt was suspended in acetone (100 mL) and daunorubicin 14-bromide/chloride (0.50 g, 0.75 mmol) was added. The resulting suspension was heated at reflux for 12 h, then filtered, and the solvent evaporated. The resulting solid was purified by flash chromatography (gradient from 9 / 1 to 8 / 2 CH_2Cl_2 / MeOH) to give a red solid that was suspended in THF dry and 1.5 M HCl in dry dioxan (0.27 mL, 0.41 mmol) was added. The suspension was stirred overnight then filtered and the precipitate washed with Et_2O and desiccated. Yield 35 %; ^{1}H-NMR ($(CD_3)_2SO$): 1.19 (d, 3H), 1.68 (m, 1H), 1.90 (m, 1H) 2.08 (m, 1H), 2.32 (m, 1H); 2.90 (d, 1H), 3.08 (d, 1H), 3.37 (m, 1H), 3.61 (m, 1H), 3.82 (s, 3H), 3.98 (s, 3H), 4.26 (q, 1H), 4.95 (m, 1H), 5.29-5.41 (m, 3H), 6.57 (d, 1H), 6.82 (d, 1H), 7.15 (m, 1H), 7.35 (m, 1H), 7.63 (m, 2H), 7.90 (m, 2H), 8,32 (s, 1H); MS (ESI^+) *m/z* 720 $(M+1)^+$.

Biochemical Methods

Reagents. Fetal bovine serum (FBS) and culture medium were supplied by Invitrogen Life Technologies (Carlsbad, CA); plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA); the protein content of cell monolayers and lysates was assessed with the BCA kit from Sigma Chemical Co (St.Louis, MO). Materials not specified were purchased from Sigma Chemical Co.

Cells. Rat cardiomyocytes H9c2 cells were grown in DMEM medium, human breast cancer cells MCF7 and MDA-MB-231 were grown in RPMI medium. All media were supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin and 1% v/v L-glutamine. Cell cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Extracellular lactate dehydrogenase (LDH) activity. To verify the cytotoxic effect of DOX, the extracellular medium was centrifuged at 12,000 × g for 15 min to pellet cell debris, whereas cells were washed with fresh medium, detached with trypsin/EDTA, re-suspended in 0.2 mL of 82.3 mmol/L triethanolamine phosphate-HCl (pH 7.6) and sonicated on ice with two 10 s bursts. LDH activity was measured in the extracellular medium and in the cell lysate, following a published method.[2] The reaction was monitored for 6 min, measuring absorbance at 340 nm with Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT) and was linear throughout the measurement time. Both intracellular and extracellular enzyme activities were expressed in μmol NADH oxidized/min/dish; extracellular LDH activity was then calculated as a percentage of the total (intracellular + extracellular) LDH activity in the dish.

Intracellular doxorubicin accumulation and efflux. The amount of DOX in whole cell lysates was measured fluorimetrically as described elsewhere,[2] using a PerkinElmer LS-5 spectrofluorimeter (PerkinElmer, Waltham, MA). Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank containing no cells was prepared for each set of experiments and its fluorescence was subtracted from that measured in each sample. Fluorescence was converted into nmol DOX/mg cell proteins using a previously-prepared calibration curve. Kinetic parameters were calculated as described elsewhere.[2] Values were fitted to Michaelis-Menten equation to calculate V_{max} and K_m, using the Enzfitter software package(Biosoft Corporation, Cambridge, United Kingdom).

In vitro topoisomerase assay. The *in vitro* activity of topoisomerase II was measured using the Topoisomerase II Drug Screening Kit (Topogen Inc, Port Orange, FL), following the manufacturer's instructions, as reported previously.[3]

Cell fractioning. Nuclear extracts were prepared according to[4]; mitochondria isolation was performed as described earlier.[5] A 50 μL aliquot of nuclear and mitochondrial extracts was sonicated and used to measure the protein content; the remaining sample was used to measure the amount of doxorubicin (as described above) or the amount of ROS (as detailed below).

Western blot analysis. Cells were treated with boiling 0.5 mL lysis buffer (10 mmol/L Tris, 100 mmol/L NaCl, 20 mmol/L KH_2PO_4, 30 mmol/L EDTA, 1 mmol/L EGTA, 250 mmol/L sucrose, pH 7.5). After sonication 1 mmol/L $NaVO_4$, 1 mmol/L NaF, 10 mmol/L dithiothreitol and the inhibitor cocktail set III (100 mmol/L AEBSF, 80 mmol/L aprotinin, 5 mmol/L bestatin, 1.5 mmol/L E-64, 2 mmol/L leupeptin and 1 mmol/L pepstatin; Calbiochem, La Jolla, CA) were added and cell lysates were centrifuged at 13,000×g for 15 min. 30 μg whole cell proteins were separated by SDS-PAGE and probed with the following antibodies: anti-Pgp (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MRP1 (Abcam, Cambridge, MA), anti-BCRP (Santa Cruz Biotechnology), anti-actin (Sigma Chemical Co.). After overnight incubation, the membrane was washed with PBS-Tween 0.1% v/v and subjected for 1 h to a peroxidase-conjugated secondary antibody (diluted 1:3000 in PBS-Tween with milk 5% w/v). The membrane was washed again with PBS-Tween, and proteins were detected by enhanced chemiluminescence (Immun-Star, Bio-Rad).

ROS measurement. Cells were rinsed with PBS and loaded with 10 μmol/L 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester (DCFDA-AM) for 10 min at 37°C, then washed and re-suspended in 0.5 mL PBS. A 50 μL aliquot of samples was sonicated and used to measure the protein content. Intracellular fluorescence of DCFH was detected using a Synregy^{TM} HT spectrofluorimeter (Bio-Tek Instruments). Excitation and emission wavelengths were 490 and 530 nm, respectively. A blank with no cells was prepared for each set of experiments, and its fluorescence was subtracted from that measured in each sample. Fluorescence was converted into nmol/mg cell proteins following the titration curve. The same procedure was followed to measure ROS on isolated mitochondria; fluorescence was converted into nmol/mg mitochondrial proteins.

Lipid peroxidation. Cells were washed with fresh medium, detached and re-suspended in 1 mL of PBS. Lipid peroxidation was detected by measuring the intracellular level of MDA with the Lipid Peroxidation Assay kit (Oxford Biomedical Research, Oxford, MI), which exploits the reaction of *N*-methyl-2-phenylindole with MDA, in the presence of hydrochloric acid, that yields a stable

chromophore with maximal absorbance at 586 nm. The absorbance was measured with a Packard EL340 microplate reader (Bio-Tek Instruments). Results are expressed as nmol/mg cell proteins, following a previously-prepared titration curve.

Statistical Analysis. All data in text and figures are given as means ± SD. The results were analyzed by one-way analysis of variance (ANOVA) and Tukey's test. $p < 0.05$ was considered significant.

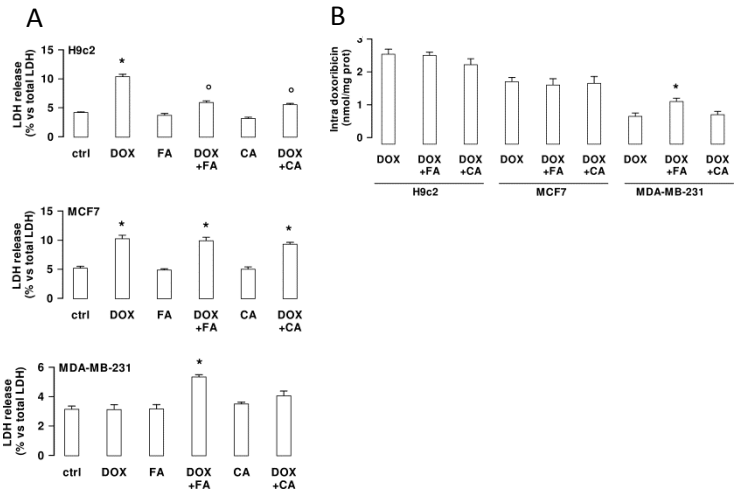


Figure S1. Cytotoxicity and intracellular doxorubicin accumulation of DOX in combination with ferulic acid (FA) or caffeic acid (CA). Cells were incubated for 24 h in fresh medium (ctrl) or with 5 µmol/L DOX, FA and CA, alone or in combination. **A**: Release of LDH was evaluated in duplicate in cell culture supernatants and in the cell lysate, and the former was expressed as percentage of the total LDH activity (extracellular + intracellular). Data are presented as means ± SD (n = 4). *Vs.* ctrl: **p* < 0.01; DOX+FA/DOX+CA *vs.* DOX: °*p* < 0.005. **B**: The amount of intracellular DOX was measured in duplicate. Data are presented as means ± SD (n = 3). DOX+FA *vs.* DOX: **p* < 0.05.

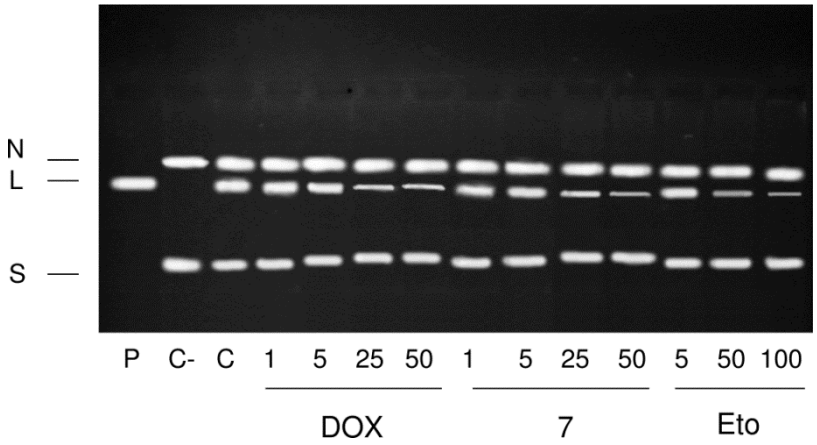


Figure S2. In vitro topoisomerase II inhibition by DOX and 7. The activity of human purified topoisomerase II was measured after incubating the enzyme with the supercoiled pHOT1 plasmid, in the absence (C) or presence of DOX (1, 5, 25, 50 µmol/L) or 7 (1, 5, 25, 50 µmol/L). Etoposide (5, 50, 100 µmol/L, Eto) was used as positive control of topoisomerase inhibition. The reaction products were resolved on agarose gel. Linear pHOT1 (lane P) was used as a marker. To obtain a blank, supercoiled pHOT1 was incubated in the absence of topoisomerase II (lane C-). N: nicked pHOT1; L: linear pHOT1; S: supercoiled pHOT1.

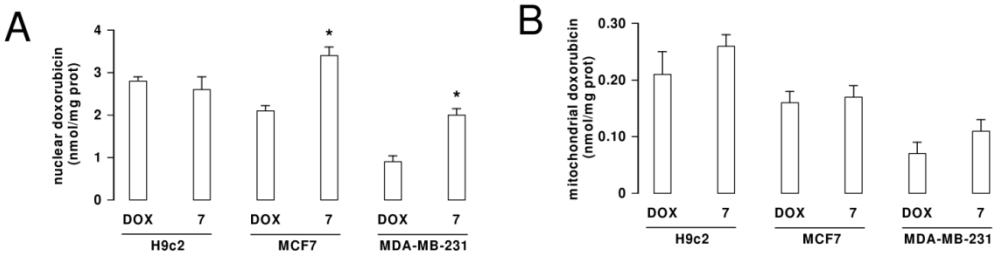


Figure S3. Intranuclear and intramitochonadrial accumulation of DOX and **7**. Cells were incubated for 24 h with 5 µmol/L DOX or **7**. **A**:The amount of doxorubicin was measured fluorimetrically in nuclear extracts in duplicate. Data are presented as as means ± SD (n = 3). *Vs.* DOX: **p* < 0.01. **B**: The amount of doxorubicin was measured fluorimetrically in mitochondrial extracts in duplicate. Data are presented as as means ± SD (n = 3).

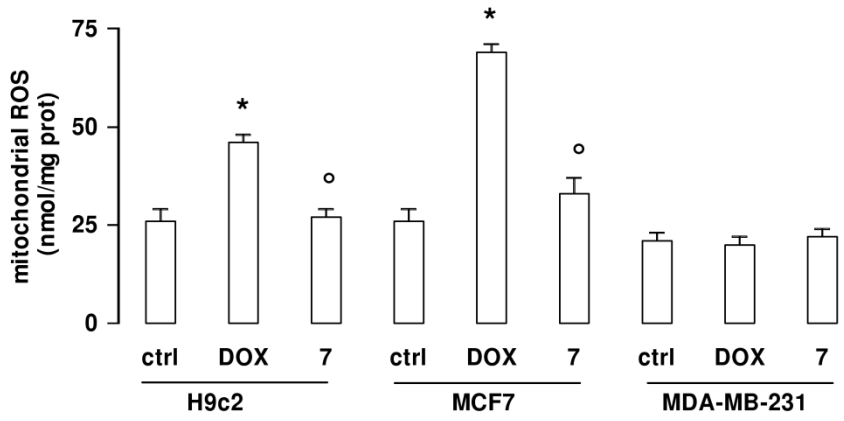


Figure S4. Intramitochonadrial ROS levels. The amount of ROS was measured in duplicate in freshly-prepared mitochondrial extract of cells grown 24 h in fresh medium (ctrl), 5 µmol/L DOX or **7.** Data are presented as as means ± SD (n = 3). *Vs.* ctrl: **p* < 0.005; **7** vs DOX: °*p* < 0.01.

References and notes

1. Fernandez, A.-M.; Dubois, V. Method for the synthesis of antracycline-peptide conjugates, WO 2004/011033, **2004**.
2. Riganti, C.; Miraglia, E.; Viarisio, D.; Costamagna, C.; Pescarmona, G.; Ghigo, D.; Bosia, A. Nitric oxide reverts the resistance to doxorubicin in human colon cancer cells by inhibiting the drug efflux. *Cancer Res.* **2005,** *65*, 516.
3. a) Riganti, C.; Rolando, B.; Kopecka, J.; Campia, I.; Chegaev, K.; Lazzarato, L.; Federico, A.; Fruttero, R.; Ghigo, D. Mitochondrial-targeting nitrooxy-doxorubicin: a new approach to overcome drug resistance. *Mol Pharm.* **2013**; *10*, 161; b**)** Riganti, C.; Rolando, B.; Kopecka, J.; Campia, I.; Chegaev, K.; Lazzarato, L.; Federico, A.; Fruttero, R.; Ghigo, D. *Mol. Pharmaceutics.* 2012, *10(1)*, 161.
4. Riganti, C.; Orecchia, S.; Pescarmona, G.; Betta, P.G.; Ghigo, D.; Bosia, A. Statins revert doxorubicin resistance via nitric oxide in malignant mesotelioma. *Int. J. Cancer* **2006,** *119*, 17.
5. Campia, I.; Lussiana, C.; Pescarmona, G.; Ghigo, D.; Bosia, A.; Riganti, C. Geranylgeraniol prevents the cytotoxic effects of mevastatin in THP-1 cells, without decreasing the beneficial effects on cholesterol synthesis. *Br. J. Pharmacol.* **2009,** *158*, 1777.