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Overcoming multidrug resistance by targeting mitochondria with NO-donating doxorubicins

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ABSTRACT

A library of nitric oxide-donor doxorubicins (NO-DOXOs) was synthesized by linking appropriate NO-donor moieties at C-14 position through an ester bridge. Their hydrolytic stability was evaluated. The intracellular accumulation and cytotoxicity of these novel NO-DOXOs were studied in DOXO-sensitive (HT29) and DOXO-resistant (HT29/dx) tumor-cells. Hydrolytically-stable compounds accumulated in HT29 and HT29/dx cells, thanks to the nitration of plasma-membrane efflux transporters. Surprisingly, no close correlation was found between intracellular accumulation and cytotoxicity. Only compounds with high mitochondria retention (due to nitration of mitochondrial efflux transporter) exert high cytotoxicity, through the activation of a mitochondrial-dependent apoptosis.

1. Introduction

Doxorubicin (DOXO) (**1**) (Fig. 1), an antitumor antibiotic isolated from cultures of *Streptomices peucetius*, is one of the most important drugs in the fight against cancer, being very effective against a wide spectrum of malignancies. The classical mechanisms underlying the antitumor effects of DOXO imply DNA modification and oxidative stress induction, but other mechanisms of action have also been proposed.[1-3] The clinical use of DOXO is limited by its cumulative and dose-dependent cardiotoxicity, and by resistance development through different mechanisms. Among these latter, a paramount role is exerted by the overexpression in cancer cells of ATP-binding cassette (ABC) transporter proteins induced by the drug. The principal pumps involved in DOXO resistance are P-glycoprotein (P-gp or ABCB1), MDR associated protein MRP1, and breast cancer resistant protein (BCRP/ABCG2). Under the action of these transporters, the drug is extruded from the cancer cell, thus limiting its cellular accumulation and consequently its toxic action. This kind of resistance is largely involved in the cross-resistance (multidrug resistance, MDR) of malignancies towards several antitumoral drugs.[4] It has been shown that nitric oxide (NO) released by several NO-donors is able to nitrate tyrosine residues of Pgp and MRPs pumps; the consequent decrease of their activity allows the increase of DOXO accumulation in the cancer cells, with a resulting increase in its toxicity.[5,6] On these foundations, two new DOXO derivatives were recently designed in which either nitrooxy (**2**) or furoxan (**3**) NO-donor moieties were combined through an ester linkage at C-14 of the antibiotic (Fig. 1). Compound **2** appeared to be an interesting lead as it was more cytotoxic than DOXO in some DOXO-resistant human cancer cell lines.[7,8] It was able to localize in the mitochondria, where it induced nitration and inhibition of the mitochondria-associated ABC transporters, decreased the flux through the tricarboxylic acid cycle, slowed down the activity of the complex I, lowered ATP synthesis, induced oxidative and nitrosative stress, and elicited release of cytochrome c and activation of the caspases 9 and 3.[8]

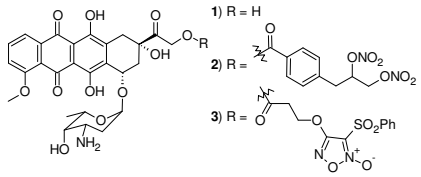


Figure 1. Doxorubicin and NO-donor DOXOs.

In order to extend this new class of semisynthetic DOXOs a small library of NO-donor DOXOs was designed and synthesized. These compounds were obtained by linking, at the C-14 position of DOXO, substructures able to release NO via different mechanisms. Compounds **12**-**17** contain nitrooxy groups, known to release NO under enzymatic catalysis. A

number of enzymes have been proposed to be implicated in this transformation, among them mitochondrial aldehyde dehydrogenase (mtALDH).[9] In fact, under the action of mtALDH, organic nitrates can be converted to nitrite, which is metabolized further to generate NO.[9] Compound **18** contains the furoxan ring, which is able to release NO under the action of thiol cofactors when suitably substituted.[10] Finally, in compound **19** the carbonyloxymethyl bridge can rapidly be hydrolyzed under physiological conditions to afford the 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate, which is a spontaneous NO-donor.[11] The hydrolytic stability in human serum of the compounds developed was evaluated. The toxicity of the novel NO-donor DOXOs against human DOXO-sensitive colon cancer HT29 cells and against their DOXO-resistant counterpart HT29/dx cells, and the localization and putative mechanisms of toxicity of the most effective compounds in the DOXO-resistant cells, were studied.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. Chemistry

^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvance 300, at 300 and 75 MHz, respectively, using $SiMe_4$ as internal standard. The following abbreviations indicate peak multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet, bs = broad singlet. Low-resolution mass spectra were recorded with a Thermo Finnigan LTQ (Ion Trap). ESI spectra were recorded on Micromass Quattro API micro (Waters Corporation, Milford, MA, USA) mass spectrometer. Data were processed using a MassLynxSystem (Waters). Melting points were determined with a capillary apparatus (Büchi 540) in open capillary. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM). The progress of the reactions was followed by thin-layer chromatography (TLC) on 5 × 20 cm plates Merck Kieselgel 60 F254, with a layer thickness of 0.20 mm. Anhydrous sodium sulfate (Na_2SO_4) was used as drying agent for the organic phases. Organic solvents were removed under reduced pressure at 30°C. Synthetic-purity solvents dichloromethane (DCM), methanol (MeOH), diethyl ether (Et_2O) and dimethylformamide (DMF) were used without purification. Dry tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone under positive N_2 pressure. Dry DMF was obtained through storage on molecular sieves 4Å. Commercial starting materials were purchased from Sigma-Aldrich, Alfa Aesar, and TCI Europe. The purity of obtained compound was determined by HPLC techniques (see supporting information).

2.1.2. General procedure for the synthesis of NO-donor doxorubicins 12-19

To the solution of 14-bromo/chloro daunorubicin hydrobromide (0.30 g, 0.45 mmol) and appropriate acid (1.35 mmol) in dry DMF KF (0.16 g, 2.7 mmol) was added in one portion. The reaction was stirred at r.t. until completed (TLC control). Solvent was removed under reduced pressure at 30°C and the resulting mixture was separated by flash chromatography (eluent: gradient from 98/2 to 80/20 DCM/MeOH) to give a red solid. The obtained compound was dissolved/suspended in dry THF and 2 equivalents of HCl 1.7 M solution in dry dioxane was added. Resulting mixture was stirred for 2 h at r.t., then diluted with *i*-Pr_2O and the precipitate was filtered, extensively washed with Et_2O and dried in desiccators to give a title compound as a red powder.

2.1.3. Daunomycin-14-yl 4-[3-(nitrooxy)propyl]benzoate (12)

Reaction time: 1.5 h, red powder (50%). Mp: 155-159°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.20 (d, 3H, $^{6'}CH_3$), 1.70 (m, 1H), 1.87 – 2.13 (m, 4H), 2.34 (m, 1H) ($^{2'}CH_2$ + $CH_2CH_2ONO_2$ + $^{8}CH_2$), 2.78 (t, 2H, $CH_2C_6H_4$), 2.90 (d, 1H), 3.12 (d, 1H) ($^{10}CH_2$), 3.61 (m, 1H, $^{4'}CH$), 3.99 (s, 3H, OCH_3), 4.28 (q, 1H, $^{5'}CH$), 4.54 (t, 2H, CH_2ONO_2), 4.96 (m, 1H, ^{7}CH); 5.31 (m, 1H, C*H*); 5.40 – 5.46 (m, 3H, $^{14}CH_2$ + $^{1'}CH$), 5.79 (br. s, 1H, O*H*), 7.43 (d, 2H), 7.67 (m, 1H), 7.92 (m, 4H) (7C*H* Ar), 13.25 (s, 1H, O*H*); 14.02 (s, 1H, O*H*); MS (ESI+): *m/z* 751 (*M*+H)$^+$.

2.1.4. Daunomycin-14-yl 4-[2,3-bis(nitrooxy)propoxy]benzoate (13)

Reaction time: 2 h, red powder (21%). Mp: 148-150°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ =1.19 (d, 3H, $^{6'}CH_3$), 1.70 (m, 1H), 1.91 (m, 1H) ($^{2'}CH_2$), 2.10 (m, 1H), 2.33 (m, 1H) ($^{8}CH_2$), 2.92 (d, 1H), 3.12 (d, 1H) ($^{10}CH_2$), 3.62 (m, 1H, $^{4'}CH$), 3.98 (s, 3H, OCH_3), 4.27 (q, 1H, $^{5'}CH$), 4.44 (dd, 1H), 4.54 (dd, 1H) ($CH_2OC_6H_4$), 4.92 – 5.11 (m, 3H, ^{7}CH + CH_2ONO_2), 5.31 (m, 1H, C*H*), 5.37 – 5.53 (m, 3H, $^{14}CH_2$ + $^{1'}CH$), 5.77 (s, 1H, OH), 5.84 (m, 1H, C*H*ONO_2), 7.12 (d, 2H), 7.65 (m, 1H), 7.94 (m, 4H) (7C*H* Ar), 13.25 (s, 1H, OH); MS (ESI+): *m/z* 828 (*M*+H)$^+$.

2.1.5. Daunomycin-14-yl 4-[3-(nitrooxy)propoxy]benzoate (14)

Reaction time: 3 h, red powder (36%). Mp: 163-166°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.14 (d, 3H, $^{6'}CH_3$), 1.70 (m, 1H), 1.85 (m, 1H) ($^{2'}CH_2$), 2.02 – 2.17 (m, 3H), 2.31 (m, 1H) ($CH_2CH_2ONO_2$ + $^{8}CH_2$), 2.86 (d, 1H), 3.06 (d, 1H) ($^{10}CH_2$), 3.55 (m, 1H, $^{4'}CH$), 3.93 (s, 3H, OCH_3), 4.12 (t, 2H, $CH_2OC_6H_4$), 4.22 (q, 1H, $^{5'}CH$), 4.65 (t, 2H, CH_2ONO_2), 4.92 (m, 1H, ^{7}CH), 5.25 (m, 1H, C*H*), 5.30 – 5.47 (m, 3H, $^{14}CH_2$ + $^{1'}CH$), 5.70 (br. s, 1H, O*H*), 7.03 (d, 2H), 7,60 (m, 1H), 7,90 (m, 4H) (7C*H* Ar), 13,19 (s, 1H, O*H*); MS (ESI+): *m/z* 767 (*M*+H)$^+$.

2.1.6. Daunomycin-14-yl 5,6-bis(nitrooxy)hexanoate (15)

Reaction time: 2 h, red powder (22%). Mp: 102°C changes shape, 105-110°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.16 (d, 3H, $^{6'}CH_3$), 1.66 – 1.90 (m, 6H) ($^{2'}CH_2$ + $2CH_2$), 2.03 (m, 1H), 2.29 (m, 1H) ($^{8}CH_2$), 2.47 (m, 2H, CH_2COO), 2.85 (d, 1H), 3.06 (d, 1H) ($^{10}CH_2$), 3.50 (m, 1H, $^{4'}CH$), 3.98 (s, 3H, OCH_3), 4.24 (q, 1H, $^{5'}CH$), 4.72 (dd, 1H), 4.92 – 4.97 (m, 2H), (CH_2ONO_2 + ^{7}CH), 5.16 – 5.32 (m, 3H, $^{14}CH_2$ + C*H*), 5.44 (m ,1H, $^{1'}CH$), 5.73 (br. s, 1H, O*H*), 7.63 (m, 1H), 7.91 (m, 2H) (3C*H* Ar), 13.22 (s, 1H, O*H*), 14.01 (s, 1H, O*H*); MS (ESI+): *m/z* 764 (*M*+H)$^+$.

2.1.7. Daunomycin-14-yl 6-nitrooxyhexanoate (16)

Reaction time: 2 h, red powder (27%). Mp: 140-144°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.16 (d, 3H, $^{6'}CH_3$), 1.41 (m, 2H), 1.55 – 1.73 (m, 5H), 1.90 (m, 1H), 2.04 (m, 1H), 2.29 (m, 1H) ($^{2'}CH_2$ + $^{8}CH_2$ + $3CH_2$), 2.43 (t, 2H, CH_2COO), 2.86 (d, 1H), 3.05 (d, 1H) ($^{10}CH_2$), 3.59 (m, 1H, $^{4'}CH$), 3.98 (s, 3H, OCH_3), 4.24 (q, 1H, $^{5'}CH$), 4.51 (t, 2H, CH_2ONO_2), 4.93 (m, 1H, ^{7}CH), 5.14 – 5.30 (m, 3H, $^{14}CH_2$ + C*H*), 5.48 (m ,1H, $^{1'}CH$), 5.70 (s, 1H, O*H*), 7.66 (m, 1H), 7.91 (m, 2H) (3C*H* Ar), 13.23 (s, 1H, O*H*), 14.02 (s, 1H, O*H*); MS (ESI+): *m/z* 703 (*M*+H)$^+$.

2.1.8. Daunomycin-14-yl [2,2-dimethyl-3-nitrooxy] propanoate (17)

Reaction time: 24 h, red powder (28%). Mp: 170-175°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.16 (d, 3H, $^{6'}CH_3$), 1.28 (s, 6H, $2CH_3$), 1.62 (m, 1H), 1.83 (m, 1H) ($^{2'}CH_2$), 2.04 (m, 1H), 2.29 (m, 1H) ($^{8}CH_2$), 2.81 (d, 1H), 3.04 (d, 1H) ($^{10}CH_2$), 3.50 (m, 1H, $^{4'}CH$), 3.77 (s, 1H), 3.97 (s, 3H, OCH_3), 4.20 (q, 1H, $^{5'}CH$), 4.65 (s, 2H, CH_2ONO_2), 4.92 (m, 1H, ^{7}CH), 5.27 (m, 3H, $14CH_2$ + C*H*), 5.57 (m, 1H, $^{1'}CH$), 7.63 (m, 1H), 7.89 (m, 2H) (3C*H* Ar.). MS (ESI+): *m/z* 689 (*M*+H)$^+$.

2.1.9. Daunomycin-14-yl [4-[(4-phenylsulfonyl)furoxan-3-yl]oxy] benzoate (18)

Reaction time: 24 h, red powder (56%). Mp: 183°C changes color, 185-190°C dec. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.21

(d, 3H, $^{6'}CH_3$), 1.71 (m, 1H), 1.91 (m, 1H) ($^{2'}CH_2$), 2.12 (m, 1H), 2.37 (m, 1H) ($^{8}CH_2$), 2.91 (d, 1H), 3.14 (d, 1H) ($^{10}CH_2$), 3.61 (m, 1H, $^{4'}CH$), 3.99 (s, 3H, OCH_3), 4.29 (q, 1H, $^{5'}CH$), 4.97 (m, 1H, ^{7}CH), 5.32 (br. s, 1H, $^{1'}CH$), 5.52 (dd, 2H, $^{14}CH_2$), 5.81 (s, 1H, C*H*), 7.63 – 8.16 (m, 12H, C*H* Ar), 13.26 (s, 1H, O*H*), 14.03 (s, 1H, O*H*); MS (ESI+): *m/z* 888 $(M+H)^+$.

2.1.10. Daunomycin-14-yl (pyrrolidin-1-yl)diazen-1-ium-1,2-diolate O^2-methylene benzene dicarboxilate (19)

Reaction time: 24 h, red powder (66%). Mp: 165°C dec. without melting. ^{1}H NMR (300 MHz, DMSO-*d6*): δ=1.20 (d, 3H, $^{6'}CH_3$), 1.71 (m, 1H), 1.90 (m, 5H) ($^{2'}CH_2$ + $2CH_2$), 2.12 (m, 1H), 2.33 (m, 1H) ($^{8}CH_2$), 3.48 (m, 4H, $2CH_2$), 3.59 (m, 1H, $^{4'}CH$), 3.99 (s, 3H, OCH_3), 4.28 (q, 1H, $^{5'}CH$), 4.98 (m, 1H, ^{7}CH), 5.31 (br. s, 1H, $^{1'}CH$), 5.53 (m, 3H), 5.84 (s, 1H, C*H*), 6.01 (s, 2H, OCH_2O), 7.68 (m, 2H, 2C*H* Ar), 7.93 (m, 4H, 4C*H* Ar), 8.13 (m, 1H, C*H* Ar), 13.27 (s, 1H, O*H*), 14.05 (s, 1H, O*H*); MS (ESI+): *m/z* 835 $(M+H)^+$.

2.2 Chemicals

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 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). DOXO and reagents not specified were from Sigma Chemical Co. Each compound was dissolved in dimethyl sulfoxide (DMSO); this stock solution was diluted in culture medium to reach a 5 µM final concentration. In each experimental condition, the concentration of DMSO in the culture medium was 1%. Control cells were treated with 1% DMSO. Preliminary experiments showed that cells treated with 1% DMSO did not differ from cells treated with culture medium without DMSO in any biological assay (data not shown).

2.3 Cell lines

Human colon cancer DOXO-sensitive HT29 cells were cultured in RPMI 1640 medium. A subpopulation of DOXO-resistant HT29 cells, named HT29/dx, was created as reported elsewhere[5] and then cultured in RPMI 1640 medium containing 200 nmol/L DOXO. Compared to HT29 cells, HT29/dx have higher expression of Pgp, MRP1 and BCRP.[7] H9c2 cardiomyocytes were cultured in DMEM medium. The culture mediums were supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine. Cell cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2.

2.4 Nitrite production

HT29 and HT29-dx cells were cultured for 24 h in fresh medium (CTRL) or in the presence of 5 µmol/L DOXO or of its NO-releasing derivatives. The amount of extracellular nitrite was measured spectrophotometrically, by adding 0.15 mL of cell culture medium to 0.15 mL of Griess reagent in a 96-well plate. After 10 min incubation at 37°C in the dark, the absorbance was detected at 540 nm with a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Winooski, VT). For each experiment, a blank was prepared in the absence of cells, and its absorbance was subtracted from that measured in the presence of cells. Nitrite concentration was expressed as nmol nitrite/mg cell proteins.

2.5 Intracellular doxorubicin accumulation

The amount of DOXO in whole-cell lysates and in mitochondrial extracts was measured spectrofluorimetrically, as described elsewhere[5] using a Synergy HT Multi-Detection Microplate Reader. Excitation and emission wavelengths were 475 and 553 nm, respectively. A blank was prepared in the absence of cells for each set of experiments, and its fluorescence was subtracted from that measured in the presence of cells. Fluorescence was converted to nmol DOXO/mg cell proteins using a calibration curve prepared previously.

2.6 Western blot analysis

Plasma-membrane-extracts were isolated by the biotination method, using the Cell Surface Protein isolation kit (Thermo Fisher Scientific Inc., Waltham, MA), as reported elsewhere[12] using an anti-pancadherin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) to check equal protein loading. Mitochondrial extracts were prepared as reported elsewhere,[8] using an anti-porin (Abcam) antibody to check equal loading of proteins. 50 µg proteins from plasma-membrane or 10 µg proteins from mitochondrial extracts were separated by SDS-PAGE and probed with the following antibodies: anti-Pgp (Santa Cruz Biotechnology), anti-MRP1 (Abcam, Cambridge, UK), and anti-BCRP (Santa Cruz Biotechnology). To analyze the presence of nitrated proteins, the mitochondrial extract was subjected to immunoprecipitation using a rabbit polyclonal anti-nitrotyrosine antibody (Millipore, Bedford, MA). Immunoprecipitated proteins were separated by SDS-PAGE and probed with anti-Pgp, anti-MRP1, and anti-BCRP antibodies. After overnight incubation, the membrane was washed with PBS-Tween 0.1% v/v and treated for 1 h with a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). The membrane was washed with PBS-Tween 0.1% v/v, and proteins were detected by enhanced chemiluminescence (Immun-Star, Bio-Rad Laboratories).

2.7. Extracellular LDH activity

To verify the cytotoxic effect of DOXO, the extracellular medium was centrifuged at 12,000 × g for 5 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 mmol/L triethanolamine phosphate-HCl (pH 7.6) and sonicated on ice with two 10 s bursts. LDH activity was measured in extracellular medium and cell lysate, as reported elsewhere.[8] The reaction was monitored for 6 min, measuring absorbance at 340 nm with a Synergy HT Multi-Detection Microplate Reader, and was linear throughout the measurement time. Both intracellular and extracellular enzyme activities were expressed in µmol NADH oxidized/min/dish, and extracellular LDH activity was calculated as the percentage of the total LDH activity occurring in the dish.

2.8. Intramitochondrial doxorubicin accumulation

HT29 and HT29-dx cells were incubated for 24 h with 5 µmol/L DOXO or its NO-releasing derivatives. Mitochondrial fractions were isolated as described elsewhere.[8] To confirm the presence of mitochondrial proteins in the extracts, 10 µg of each sonicated sample were subjected to SDS-PAGE and probed with an anti-porin antibody (Abcam; data not shown). The amount of intramitochondrial DOXO was measured spectrofluorimetrically as described above. The results were expressed as nmol DOXO/mg mitochondrial cell proteins.

2.9. Caspase activity

HT29 and HT29-dx cells were cultured for 24 h in fresh medium (CTRL), in the presence of 5 µmol/L DOXO, or of its NO-releasing derivatives. Cells were lysed in 0.5 mL of caspase lysis buffer (20 mmol/L Hepes/KOH, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethanesulfonyl fluoride, 10

µg/mL leupeptin, pH 7.5). 20 µg cell lysates were incubated for 1 h at 37°C with 20 µmol/L of the fluorogenic substrate of caspase 9 Ac-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (LEHD-AMC) or of the caspase 3 substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC), in 0.25 mL caspase assay buffer (25 mmol/L Hepes, 0.1 % w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10% w/v sucrose, 10 mmol/L dithiothreitol, 0.01% w/v egg albumin, pH 7.5). The reaction was stopped by adding 0.75 mL ice-cold 0.1% w/v trichloroacetic acid and the fluorescence of AMC fragment released by active caspases was read using a Synergy HT Multi-Detection Microplate Reader. Excitation and emission wavelengths were 380 nm and 460 nm, respectively. Fluorescence was converted into nmol AMC/µg cell proteins, using a calibration curve prepared previously with standard solutions of AMC.

2.10. Stability of compounds in human serum

The stability of the compounds (**2**, **12**-**19**) in the presence of esterase was evaluated by incubating them in human serum; the solution of compound (10 mM in DMSO) was added to human serum (sterile filtered from human male AB plasma, Sigma-Aldrich) preheated at 37 °C; the final concentration of the compound was 100 µM. The solution was incubated at 37 ± 0.5 °C, and at appropriate time intervals a 300 µL aliquot of reaction mixture was withdrawn and added to 300 µL of acetonitrile containing 0.1% HCOOH, in order to deproteinize the serum. The sample was sonicated, vortexed, and centrifuged for 10 min at 2150 × g. The clear supernatant was filtered through 0.45 µm PTFE filters (Alltech) and analyzed by RP-HPLC. All experiments were performed at least in triplicate. RP-HPLC analysis was performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with an injector (Rheodyne, Cotati, CA, USA), a quaternary pump (model G1311A), a membrane degasser (model G1379A), and a diode-array detector (DAD, model G1315B) integrated into the HP1100 system. The data were processed using a HP ChemStation system (Agilent Technologies). The analytical column was a Tracer Excel 120 ODSB (25 × 0.46, 5 µm; Tecnokroma). The mobile phase consisted of acetonitrile 0.1% HCOOH/water 0.1% HCOOH 40/60 (v/v) and had a flowrate of 1.0 mL/min. The injection volume was 20 µL. The column effluent was monitored at 234 and 480 nm, referenced against a 700 nm wavelength. By the RP-HPLC procedure, compounds and any degradation products (e.g. DOXO, aglycone, carboxylic acids bearing NO-donor moieties (**4**-**11**) were separated and quantified. The compounds were quantified using a calibration curve obtained with standard solutions chromatographed in the same experimental conditions, with a concentration range of 1−100 µM ($r^2 > 0.99$).

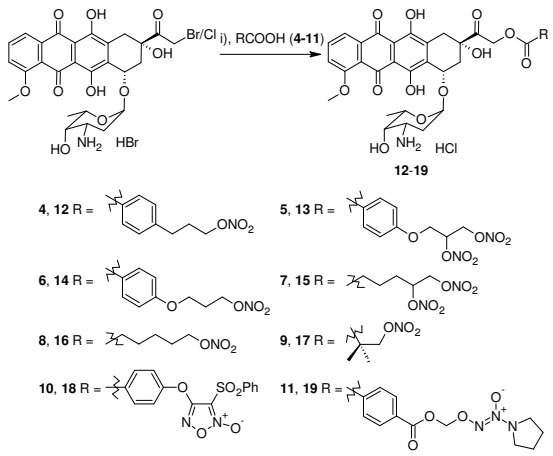
2.11. Statistical Analysis

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.

3. Results and discussion

3.1. Chemistry

Two different synthetic approaches are generally followed to prepare doxorubicin-14-yl esters: one uses acylation of the doxorubicin 14-OH group with activated carboxylic acid. This strategy requires the protection/deprotection of the amino group of the aminosugar, and can give rise to different side-products (e.g. substitution of 4'-OH group).[13] The second entails the nucleophilic substitution of daunomicin 14-Br/Cl derivative with carboxylate anions. Carboxylate can be pre-formed and used as the sodium salt, or generated *in-situ* in the presence of potassium carbonate or triethylamine. Acetone remains the solvent of choice, and a large excess of carboxylate must be used.[14] This approach presents some problems that limit its use to synthesize NO-donor DOXO: the large excess of carboxylates, their low solubility in acetone, and heating or the use of strong bases, reduce the reaction yield and make the purification step rather complex. To avoid at least some of these problems, the reaction conditions were optimized using the aprotic polar solvent DMF (Scheme 1). The carboxylates were generated *in-situ* using 2 equivalents of KF. The reaction time was highly dependent on the excess of carboxylic acid used. The optimal rate 14-Br/Cl derivative/carboxylic acid was found to be 1/3. Under these conditions, the reaction is complete in 2-24 h, and the yield ranges from 20 to 50%. This experimental procedure allowed the synthesis of a small library of NO-DOXOs (**12**-**19**) starting from carboxylic acids bearing NO-donor moieties (**4**-**11**) (Scheme 1).



Scheme 1. NO-DOXOs synthesis: i) DMF, KF, rt, then dry THF, HCl in dry dioxan.

3.2. Biological assays

The release of nitrite, the stable derivative of NO, from the synthetic DOXOs cultured with sensitive HT29 cells or with resistant HT29/dx cells, was first measured. These two cell lines were the models used to evaluate the effects of DOXO derivatives bearing nitrooxy (**2**) or furoxan (**3**) moieties.[7,8] DOXO and **2** were used as reference compounds in all assays. As shown in Figure 2, DOXO only increased NO levels in sensitive cells, whereas **2** increased NO in both sensitive and resistant cells. This result is in agreement with previous findings, showing that **2** restores the ability of MDR cells to increase NO levels in response to DOXO.[7,8] Of note, all the compounds, **12**-**19**, induced a significant increase of nitrite compared to untreated cells. Moreover, the cell culture medium of HT29/dx cells treated with compounds **12**-**19** contained significantly more nitrite than the medium of HT29/dx cells treated with DOXO (Fig. 2).

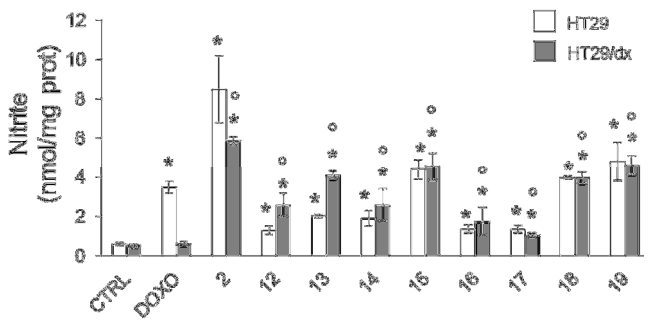


Figure 2. Nitrite levels in DOXO-sensitive and DOXO-resistant cells. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated cells (CTRL): * $p < 0.02$; for HT29/dx cells, *vs.* DOXO-treated cells: ° $p < 0.05$.

These data show that, although to different extents, all the compounds studied increased the NO concentration in HT29/dx cells better than the parent DOXO. The source of NO in cells treated with NO-releasing DOXOs is dual: NO can be released by the compound itself, or DOXO can up-regulate the inducible isoform of NO synthase (iNOS), which in turn produces large amounts of NO. This event occurs in DOXO-sensitive cells, where the drug is able to accumulate sufficiently to induce iNOS gene transcription, but not in DOXO-resistant cells, from which the drug is pumped out by ABC transporters.[12] The greater the intracellular DOXO accumulation, more NO synthesized by iNOS. The NO produced is a strong cytotoxic agent in both DOXO-sensitive and DOXO-resistant cancer cells[5,12,15] and a strong inhibitor of ABC transporters activity, thereby reversing DOXO-resistance.[5,12]

It thus appeared of interest to determine whether the different abilities to increase NO concentration of compounds **12**-**19** might produce different degrees of intracellular accumulation, and different nitration of surface ABC transporters, in HT29 vs. HT29/dx cells. Cells were cultured for 24 h in fresh medium (CTRL) or in the presence of 5 μmol/L DOXO or its NO-releasing derivatives **2**, **12**-**19**. As expected, DOXO was less retained in HT29/dx than in HT29 cells, whereas compound **2** was accumulated more than did DOXO in both HT29 and HT29/dx cells. All the compounds except **19** were retained at least as much as DOXO in HT29 and HT29/dx cells; compounds **2**, **12**, **13**, **14**, **16**, **17** accumulated to a greater extent than DOXO in HT29/dx cells (Fig. 3A). This trend was in line with the different degree of nitration of MRP1 and BCRP present on the plasma-membrane. These transporters were nitrated by DOXO, **2**, **12**, **13**, **14** and **17** in HT29 cells, and by **2**, **12**, **13**, **14**, **16** and **17** in HT29/dx cells (Fig. 3B). As already observed for this cell model, no nitration was detectable on Pgp.[5,8,12] The nitration of MRP1, which is particularly expressed in HT29/dx cells, and – to a lesser extent – that of BCRP, which is less expressed, likely explained the higher accumulation of the above mentioned compounds in DOXO-resistant cells.

It was next determined whether the greater intracellular retention due to the nitration of plasma-membrane-associated ABC transporters was correlated with higher cytotoxicity . The release of lactate dehydrogenase (LDH) in the extracellular medium, taken as index of DOXO-induced cytotoxicity and necrosis,[5] confirmed that DOXO was cytotoxic only in HT29 cells, while compound **2** was cytotoxic in both HT29 and HT29/dx cells. Surprisingly, only compounds **17** and **18** were more cytotoxic than DOXO in both HT29 and HT29/dx cells (Fig. 4).

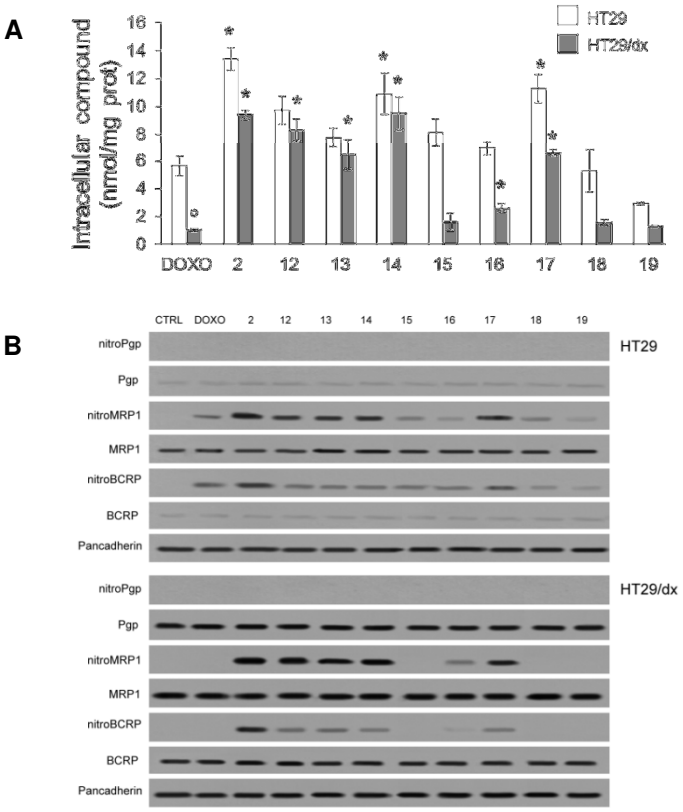


Figure 3. Intracellular accumulation of compounds **12**-**19** in HT29 and HT29/dx cells, and nitration of plasma-membrane associated ABC transporters. **A**. The amount of DOXO in whole cell lysates was measured fluorimetrically. Measurements were performed in triplicate and data are presented as means ±SD (n = 3). *Vs.* DOXO-treated HT29 or HT29/dx cells: *$p<0.001$; DOXO-treated HT29/dx cells *vs.* DOXO-treated HT29: °$p<0.001$. **B**. Western blot detection of nitrated plasma-membrane associated ABC transporters. The figure is representative of three similar experiments.

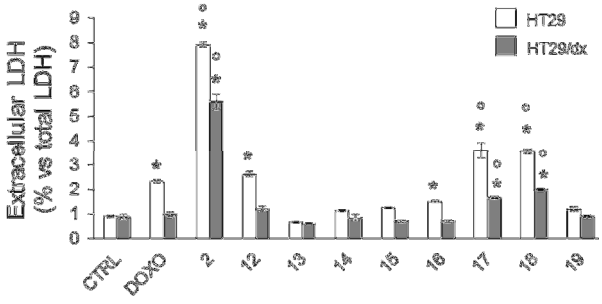


Figure 4. Cytotoxicity of compounds **12**-**19** in HT29 and HT29/dx cells. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated HT29 or HT29/dx cells (CTRL): * $p < 0.002$; *vs* DOXO-treated HT29 or HT29/dx cells: ° $p < 0.001$.

As expected, DOXO increased iNOS expression in HT29 cells, but not in HT29/dx cells (Supplementary Figure 1). Compounds **2**, **17** and **18,** which were the most cytotoxic ones (Figure 4), up-regulated iNOS at the same extent in both cell populations (Supplementary Fig. 1). These results suggest that the differences in nitrite levels (Fig. 2) were likely due to different release of NO from the compounds than to a different up-regulation of iNOS. Moreover, since only for compound **2** was there a close correlation between levels of NO produced, intracellular accumulation, nitration of surface ABC transporters,

and cytotoxic effects against DOXO-resistant cells, these data also led to the hypothesis that – alongside nitrite production, intracellular accumulation, and nitration of surface ABC transporters – the cytotoxicity of synthetic NO-releasing DOXOs might depend on additional factors.

One possible explanation for the lack of correlation between NO level, intracellular accumulation, and cytotoxicity is the different susceptibility to esterase enzymes, which gives the different compounds different hydrolytic stabilities (Table 1).

Table 1. Hydrolytic stability of NO-DOXOs in human serum

Compounds	$t_{½}$ (h) human serum esterases	Compounds	$t_{½}$ (h) human serum esterases
2	16.0	**16**	4.9
12	24.6	**17**	21.7
13	34.7	**18**	>>24h[a]
14	26.7	**19**	2.8
15	6		

[a] 73.5% conc. at 24h.

In the presence of human serum esterases compounds **12**-**18** undergo hydrolysis of the ester function, giving DOXO plus the corresponding NO-donor carboxylic acid (**4**-**10**). Conversely, compound **19** is quickly hydrolyzed at the carbonyloxymethyl bridge. Compounds obtained from the aromatic carboxylic acids **2**-**12** and **18** are generally stable to esterase action, while aliphatic acids give rise to easily hydrolyzed esters **15**, **16**. The presence of two methyl groups close to the ester function remarkably increases the stability of NO-DOXO **17**. These results could explain the lack of efficacy of compounds **15**, **16** and **19**, which had lower stability than **2**, and the good efficacy of compounds **17** and **18**, which were similar to **2** in terms of stability. However, the highly stable compounds **12**, **13** and **14** were characterized by the lowest cytotoxic efficacy in the biological assays.

Thus, neither the ability to produce NO, not that of being retained within cancer cells, nor yet the compounds' stability profile, appear to be determinant factors that induce cytotoxicity in resistant cells treated with NO-DOXOs.

MDR cells have a more active mitochondrial energy metabolism than chemosensitive cells, and are particularly susceptible to the apoptotic catastrophe induced by mitochondrial damage.[8] The preferential delivery of DOXO into the mitochondria is thus an effective strategy to overcome MDR, because mitochondrial accumulation prevents the efflux of DOXO through the ABC transporters present on the plasma membrane, and induces cell death by inhibiting the mitochondrial DNA topoisomerase 2A and damaging mitochondrial DNA.[16] Synthetic NO-DOXOs with preferential mitochondrial accumulation, like compound **2**, possess the added value of altering the mitochondrial energy metabolism, inducing oxidative/nitrosative damage, and triggering mitochondrial-dependent apoptosis.[8] It was thus decided to investigate whether the differences in the cytotoxic efficacy of the library compounds synthesized here were related to their differing accumulation within the mitochondria of DOXO-resistant cells.

In line with previous findings,[8,17] in both HT29 and HT29/dx cells compound **2** had a higher intramitochondrial accumulation than DOXO, whereas the latter was retained within the mitochondria in HT29 but not in HT29/dx cells (Fig. 5A). This pattern of accumulation was likely due to the nitration of mitochondrial ABC transporters, an event that reduces the efflux of DOXO.[5,8] Pgp, MRP1 and BCRP, the ABC transporters involved in DOXO efflux,[4] were all expressed in the mitochondria of both HT29 and HT29/dx cells; MRP1 and BCRP levels were higher in the mitochondrial extracts of the HT29/dx cells (Fig. 5B). By nitrating MRP1 and BCRP in HT29 cells, DOXO was well retained in the mitochondria of this sensitive cell population; conversely, DOXO was unable to nitrate mitochondrial ABC transporters and accumulate in the mitochondria of HT29/dx cells, where the drug was rapidly effluxed by the surface ABC transporters.[5,8,17] By contrast, compound **2**, which elicited strong nitration of mitochondrial MRP1 and BCRP in both HT29 and HT29/dx cells, accumulated in larger amounts in the mitochondria of both sensitive and resistant cells. Compound **17** and – to a lesser extent – compound **18** had more marked intramitochondrial retention than DOXO in both HT29 and HT29/dx cells (Fig. 5A): whereas **17** nitrated Pgp, MRP1 and BCRP, **18** elicited detectable nitration of MRP1 (Fig. 5B). This event appeared sufficient to increase the intramitochondrial accumulation of **18**. Compounds **12**, **13**, **14** and **19** had slight intramitochondrial retention, which was associated with the absence of nitration of mitochondrial ABC transporters (see Fig. 5B for compound **19**; data not shown for compounds **12**-**14**).

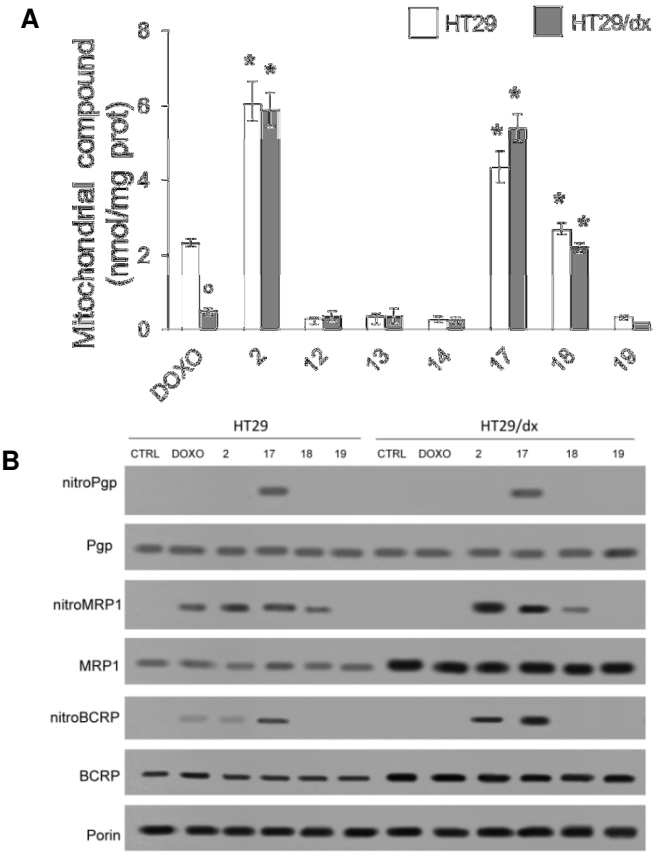


Figure 5. Intramitochondrial accumulation of compounds **12**-**19** and nitration of mitochondrial ABC transporters in HT29 and HT29/dx cells. **A**. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* DOXO-treated HT29 or HT29/dx cells: *p<0.002; HT29 *vs.*

HT29/dx cells: °p<0.001. **B**. Western blot detection of nitrated mitochondrial ABC transporters. The figure is representative of three similar experiments.

These results suggest that the cytotoxicity of the NO-releasing DOXOs is closely dependent on their ability to localize within the mitochondria. Moreover, the affinity towards mitochondrial isoform of aldehyde dehydrogenase (ALDH-2) could also play a crucial role in determining the release of NO from organic nitrates: this might also explain the differences in activities of compounds (compare for instance the nitrite levels in cells treated with **2** and **13**; Fig. 2).

The mitochondrial damage induced by the inhibition of topoisomerase 2A elicited by DOXO,[16] by the impairment of the mitochondrial energy metabolism, and by the nitrosative/oxidative stress elicited by NO,[8] resulted in the loss of cytochrome c from permeabilized mitochondria, and in the activation of caspase 9/caspase 3 axis.[18] Indeed, compounds **2**, **17** and **18** increased the activation of caspase 9 (Fig. 6A) and 3 (Fig. 6B) in both HT29 and HT29/dx cells, as did DOXO in HT29 cells. By contrast, the compounds that accumulated little within the mitochondria (i.e. **12**-**15**, **19**) did not activate the caspase 9/caspase 3 axis (Fig. 6). These data may provide a rational explanation for the different cytotoxic efficacy of the NO-releasing DOXOs of the compounds studied here: the greater the intramitochondrial accumulation (Fig. 5A), the higher was the cytotoxicity in terms of cell damage and necrosis (Fig. 4), and apoptosis (Fig. 6).

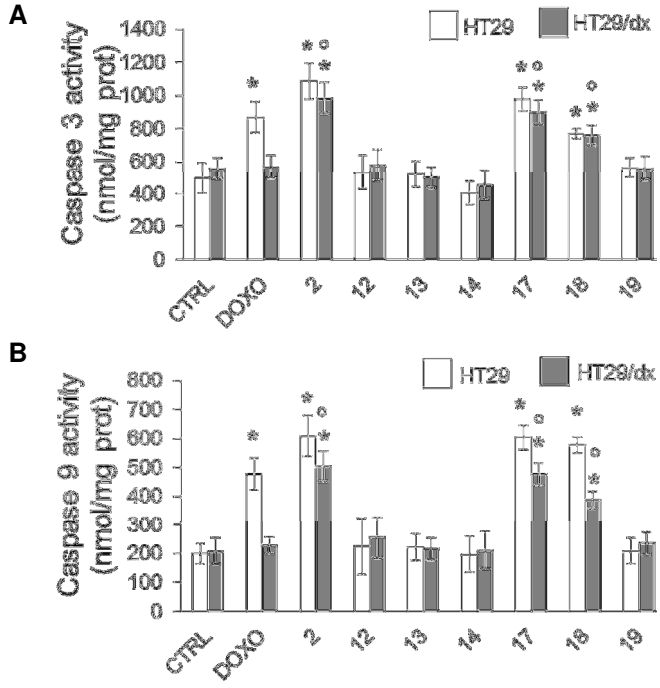


Figure 6. Activation of caspase 3 (**A**) and caspase 9 (**B**) by compounds **2**, **12**-**19** in HT29 and HT29/dx cells. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated HT29 or HT29/dx cells (CTRL): *p<0.01; for HT29/dx cells, *vs* DOXO-treated cells: °p <0.01.

Finally, since cardiotoxicity is the most common side-effect of DOXO, we verified the toxicity of the compounds in H9c2 cardiomyocytes. As expected, DOXO significantly increased cell damage (Fig. 7). The NO-donating DOXOs had a variable degree of toxicity that might be due to the different uptake and metabolism of each compound within cardiomyocytes. Of note, compounds **2, 17** and **18**, which were the most active anti-tumor compounds, did not display higher cytotoxicity than DOXO (Fig. 7). This result, although obtained in an *in vitro* system, may be important in a translational perspective, suggesting that these two compounds do not couple the higher efficacy against resistant tumors with an increased cardiotoxicity.

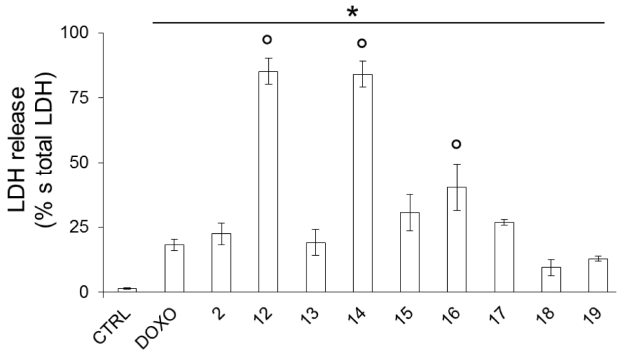


Figure 7. Cytotoxicity of compounds **12**-**19** in H9c2 cells. Measurements were performed in triplicate and data are presented as means ±SD (n = 3); *vs.* untreated cells (CTRL): * $p < 0.05$; *vs* DOXO-treated cells: ° $p < 0.05$.

Conclusions

This study has produced a small library of nitrooxy-conjugated DOXOs that were tested as possible tools to overcome DOXO-resistance in MDR human cancer cells. Some compounds (i.e. compounds **2**, **17**, **18**) were found to be more effective than DOXO against MDR cells, without showing an increased cardiotoxicity *in vitro*. Their greater cytotoxicity was due not only to the increased intracellular accumulation, by nitrating the ABC transporters present on the cell surface, but also to the drug's ability to localize within the mitochondria, by nitrating the ABC transporters present in the mitochondrial membrane. Apoptosis triggered by mitochondrial damage is responsible for the cytotoxicity of those NO-releasing DOXOs with strong tropism for mitochondria, and for their MDR-reversing properties.

Overall, this study shows that the synthetic NO-releasing drugs with physico-chemical properties and/or conjugated with specific moieties favoring intramitochondrial delivery are very effective against DOXO-resistant cells. These features should be considered in the design of future NO-releasing DOXOs as effective MDR-reversing tools.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version.

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Overcoming multidrug resistance by targeting mitochondria with NO-donating doxorubicins.

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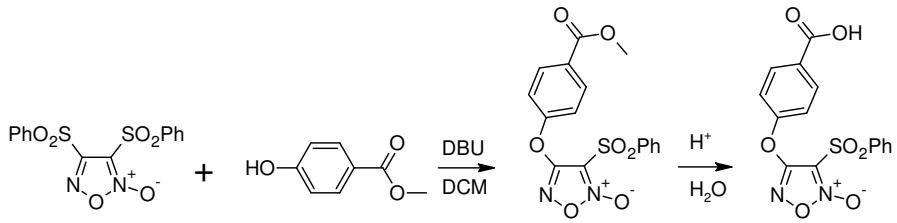
Supplementary data.

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded on a BrukerAvance 300, at 300 and 75 MHz, respectively, using $SiMe_4$ as internal standard. The following abbreviations indicate peak multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet, bs = broad singlet. Low-resolution mass spectra were recorded with a Thermo Finnigan LTQ (Ion Trap). ESI spectra were recorded on Micromass Quattro API micro (Waters Corporation, Milford, MA, USA) mass spectrometer. Data were processed using a MassLynxSystem (Waters). Melting points were determined with a capillary apparatus (Büchi 540) in open capillary. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM). The progress of the reactions was followed by thin-layer chromatography (TLC) on 5 × 20 cm plates Merck Kieselgel 60 F_{254}, with a layer thickness of 0.20 mm. Anhydrous sodium sulfate (Na_2SO_4) was used as drying agent for the organic phases. Organic solvents were removed under reduced pressure at 30°C. Synthetic-purity solvents ethyl acetate (EtOAc), acetone, hexane, dichloromethane (DCM), acetonitrile (CH_3CN), methanol (MeOH), diethyl ether (Et_2O), dimethylformamide (DMF) and 40–60 petroleum ether (PE) were used without purification. Dry tetrahydrofuran (THF), was distilled immediately before use from Na and benzophenone under positive N_2 pressure. Dry DCM was distilled from P_2O_5 and kept on molecular sieves 4Å. Dry DMF was obtained through storage on molecular sieves 4Å. Commercial starting materials were purchased from Sigma-Aldrich, Alfa Aesar, and TCI Europe. Compounds **4**, [1] **5**,[1] **6**,[1] **7**,[1] **8**[2] and **9**[3] were synthesized following methods described in the literature.

Synthesis of [4-[(3-phenylsulfonyl)furoxan-4-yl]oxy] benzoic acid (10). The following multistep procedure was used.

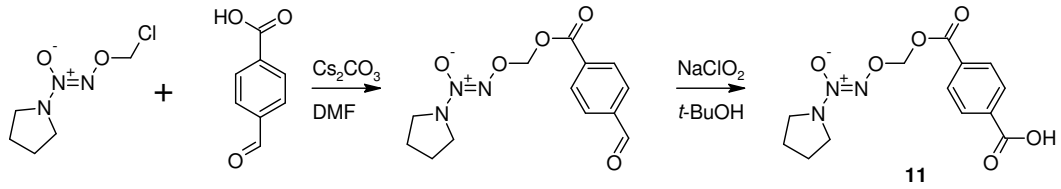


10

Methyl [4-[(3-phenylsulfonyl)furoxan-4-yl]oxy] benzoate. 3,4-Diphenylsulfonyl furoxan (6.00 g, 16.0 mmol) and methyl p-hydroxybenzoate (2.43 g, 16.0 mmol) were dissolved in DCM (50 mL). To this solution DBU (4.80 mL, 32.0 mmol) was added and the mixture was stirred at r.t. overnight. The reaction was quenched with 20 mL of H_2O and the product was extracted into DCM (3×30 mL). The organic layer was washed with NaOH 0.1 N (30 mL) and brine, dried and the solvent was removed. Obtained solid was purified by crystallization from EtOH (46%).
Mp = 127.0-128.0 °C (EtOH).
MS (CI): *m/z* 377 $(M+H)^+$.
^{1}H NMR (DMSO-d_6) δ (ppm): 3.89 (s, 3H, OCH_3); 7.60 (m, 2H, 2C*H* Ar); 7.77 (m, 2H, 2C*H* Ar); 7.92 (m, 1H, C*H* Ar); 8.06 (m, 4H, 4C*H* Ar).
^{13}C NMR (DMSO-d_6) δ (ppm): 53.3, 111.3, 119.6, 127.6, 128.5, 129.9, 131.4, 136.2, 136.7, 156.2, 157.6, 165.2.

The preceding methyl ester (1.79 g, 4.80 mmol) and p-toluensolfonic acid (11.42 g, 0.06 mmol) were dissolved under reflux in the H_2O / dioxane (25 mL / 25 mL) mixture. The solution was refluxed overnight, cooled to the r.t. and quenched with H_2O (150 mL). White precipitate formed was collected, washed with H_2O and purified by RP flash chromatography (SP1 system, Biotage AB, Sweden; RP-18 column, Biotage SNAP KP-C18-HS), with eluent TFA 0.1% in H_2O / CH_3CN, 40/60 to give a title compound as a white solid (25%).
Mp = 213.0-214.0°C (dec.).
MS (CI): *m/z* 363 $(M+H)^+$.
^{1}H-NMR (DMSO-d_6) δ (ppm): 7.58 (d, 2H, 2C*H* Ar), 7.80 (m, 2H, 2C*H* Ar), 7.95 (m, 1H, C*H* Ar), 8.06 (m, 4H, 4C*H* Ar), 13.11 (bs, 1H, COO*H*).
^{13}C NMR (DMSO-d_6) δ (ppm): 111.4, 119.5, 128.6, 128.8, 130.0, 131.6, 136.3, 136.8, 156.0, 157.7, 166.3.

Synthesis of O^2-(4-carboxybenzoyloxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (11). The following multistep procedure was used:



***O^2*-(4-formylbenzoyloxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate**. 4-formylbenzoic acid (550 mg, 3.70 mmol) was dissolved in DMF dry (5 mL) and Cs_2CO_3 (600 mg, 1.85 mmol) was added. The reaction mixture was stirred at r.t. for 30 min, then O^2-chloromethyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate[4] was added in one portion. Reaction was stirred overnight, then solvent was removed. The oily residue was quenched with $KHCO_3$ 1N (25 mL) and extracted with Et_2O (25 mL). Organic solvent was washed with brine, dried and evaporated. Obtained solid was crystallized from *i*-Pr_2O to give a title compound as a white solid (51%).
Mp = 97.5-100.0 °C (*i*-Pr_2O).
MS (CI): *m/z* 294 $(M+H)^+$.
^{1}H-NMR (DMSO-*d6*) δ (ppm): 1.87 (m, 4H, 2CH_2), 3.48 (m, 4H, 2CH_2), 6.01 (s, 2H, OCH_2O), 8.08 (d, 2H, C*H* Ar), 8.20 (d, 2H, C*H* Ar), 10.13 (s, 1H, C*H*O);
^{13}C NMR (DMSO-*d6*) δ (ppm): 22.5, 50.5, 88.1, 129.8, 130.2, 133.4, 139.6, 164.1, 193.0.

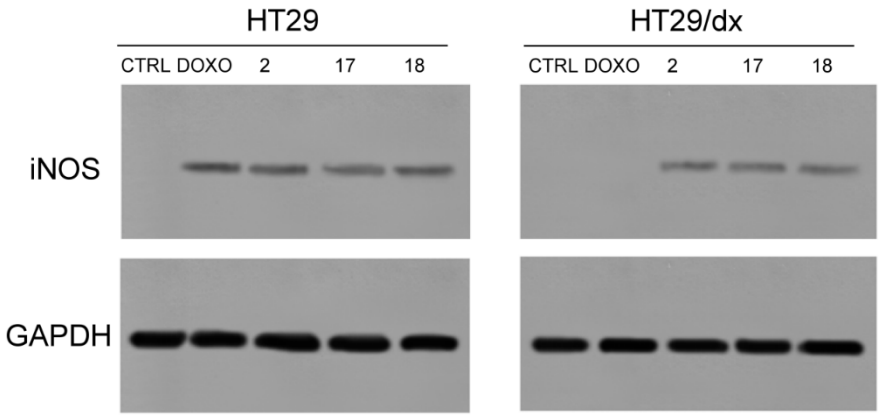
The preceding aldehyde (550 mg, 1.80 mmol) was dissolved in *t*-BuOH (10 mL) and 2-methyl-2-butene (1.91 mL, 18.0 mmol) was added, followed by 2 drops of acetic acid and the solution of $NaClO_2$ (204 mg, 2.25 mmol) in H_2O (1 mL). Reaction mixture was stirred overnight at r.t. Reaction was quenched with $KHCO_3$ 1N solution (25 mL) and water phase was washed with hexane (2×25 mL). Then HCl 1N solution (30 mL) was added and the product was extracted with DCM (2×20 mL). Organic layer was washed with brine, dried and evaporated. Obtained white solid was washed with *i*-Pr_2O, filtered and purified by crystallization from toluene (36%).
Mp = 176.5-179.0°C (toluene, dec.).
MS: (CI) *m/z* 310 $(M+H)^+$.
^{1}H-NMR (DMSO-*d6*) δ (ppm): 1.87 (m, 4H, 2CH_2), 3.49 (t, 4H, 2CH_2), 6.00 (s, 2H, OCH_2O), 8.10 (s, 4H, C*H* Ar);
^{13}C-NMR (DMSO-*d6*) δ (ppm): 22.5, 50.5, 88.0, 129.8, 132.3, 135.4, 164.2, 166.5.

iNOS expression. Cells were lysed in MLB buffer (125 mmol/L Tris-HCl, 750 mmol/L NaCl, 1% v/v NP40, 10% v/v glycerol, 50 mmol/L $MgCl_2$, 5 mmol/L EDTA, 25 mmol/L NaF, 1 mmol/L $NaVO_4$, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.5), sonicated and centrifuged at 13,000 x g for 10 min at 4°C. 20 µg of proteins from cell lysates were subjected to Western blotting and probed with the following antibodies: anti-iNOS (Cell Signaling Technology, Danvers, MA), anti-glyceraldehyde- 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The proteins were detected by enhanced chemiluminescence (Bio-Rad Laboratories).

HPLC purity evaluation: HPLC analyses were performed with a HP 1100 HPLC 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. The analytical column was a Tracer Excel 120 ODS-B (4.6 × 250 mm, 5 µm, Technocroma, Barcelona), the eluates were analyzed at λ 226 nm, 254 nm and 480 nm (vs 700 nm). 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 at flow rates of 1.0 mL·min-1. Data analysis was done using a HP ChemStation system (Agilent Technologies). The retention time and the purity of obtained compounds are reported bellow.

Compound	t_R (min)	PHPLC
12	11.7	98%
13	11.8	94%
14	11.7	96%
15	11.2	94%
16	11.2	98%
17	10.6	95%
18	11.3	99%
19	10.9	96%

Supplementary Figures



Supplementary Figure 1. iNOS expression in HT29 and HT29/dx cells after treatment with DOXO and NO-releasing DOXOs. HT29 and HT29/dx cells were incubated 24 h in fresh medium (CTRL), or in the presence of doxorubicin (DOXO), compounds **2**, **17** and **18**. The expression of iNOS was measured in whole cell lysate by Western blotting. The expression of GAPDH was used as control of equal control loading. The figure is representative of two similar experiments.

Suplementary References

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