Supporting Information

Light-Regulated NO Release as Novel Strategy to Overcome Multidrug Resistance of Doxorubicin

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Chemicals.

All chemicals were purchased by Sigma-Aldrich, Alfa Aesar, and TCI Europe and used as received. 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), dimethylformamide (DMF) and 40–60 petroleum ether (PE) were used. Dry tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone under positive N_2 pressure. Dry DMF was obtained through storage on 4Å molecular sieves. DCM was dried over P_2O_5 and freshly distilled prior to use. All solvents used for the spectrophotometric studies were spectrophotometric grade. The plastic ware for cell cultures was obtained from Falcon (Becton Dickinson, Franklin Lakes, NJ). The electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). The protein content of cell lysates was assessed with the BCA kit from Sigma Chemicals Co. (St. Louis, MO). Unless specified otherwise, all reagents were purchased from Sigma Chemicals Co.

Instrumentation

^1H 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 are used to designate peak multiplicity: *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet, *bs* = broad singlet. ESI MS spectra were recorded on a Micromass Quattro API micro (Waters Corporation, Milford, MA, USA) mass spectrometer. Data were processed using a MassLynxSystem (Waters). High resolution MS spectra were recorded on a Bruker Bio Apex Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Apollo I ESI source, a 4.7 T superconducting magnet, and a cylindrical infinity cell (Bruker Daltonics, Billerica, MA, USA). 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. UV-Vis spectra absorption and fluorescence emission spectra were recorded with a JascoV-560 spectrophotometer and a Spex Fluorolog-2 (mod. F-111) spectrofluorimeter, respectively, in air-equilibrated solutions, using either quartz cells with a path length of 1 cm. Fluorescence lifetimes were recorded

with the same fluorimeter equipped with a TCSPC Triple Illuminator. The samples were irradiated by a pulsed diode excitation source Nanoled at 455 nm. The kinetic was monotored at 595 nm and each solution itself was used to register the prompt at 455 nm. The system allowed measurement of fluorescence lifetimes from 200 ps.

Irradiation of the samples in solution was performed in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) under gentle stirring, by using a continuum laser with λ_{exc} = 405 nm (ca. 100 mW) having a beam diameter of ca. 1.5 mm.

Direct monitoring of NO release for samples in solution was performed by amperometric detection with a World Precision Instrument, ISO-NO meter, equipped with a data acquisition system, and based on direct amperometric detection of NO with short response time (< 5 s) and sensitivity range 1 nM – 20 μM. The analog signal was digitalized with a four-channel recording system and transferred to a PC. The sensor was accurately calibrated by mixing standard solutions of $NaNO_2$ with 0.1 M H_2SO_4 and 0.1 M KI according to the reaction:

$$4H^+ + 2I^- + 2NO_2^- \rightarrow 2H_2O + 2NO + I_2$$

Irradiation was performed in a thermostated quartz cell (1 cm pathlength, 3 mL capacity) using the above continuum laser with λ_{exc} = 405 nm. NO measurements were carried out under stirring with the electrode positioned outside the light path in order to avoid NO signal artefacts due to photoelectric interference on the ISO-NO electrode.

Syntheses

All syntheses were carried out under a low intensity level of visible light. **NOP1** and 14-bromo/chloro daunorubicin hydrobromide were synthesized according to previously reported procedures.[1S,2S]

DOX-NOP1 was synthesized in according with the steps reported in Scheme S1.

4-[(3-{[4-Nitro-3-trifluoromethyl-phenyl]amino}propyl)carbamoyl]benzoic acid (**NOP-Ac**). To a stirred solution of *N*-[4-nitro-3-(trifluoromethyl)phenyl]propane-1,3-diamine (1.20 g, 4.60 mmol) in dry CH_2Cl_2 (35 mL) Et_3N (0.700 mL, 5.00 mmol) was added. The mixture was cooled in the ice-bath and a solution of 4-formylbenzoyl chloride (0.850 g, 5.00 mmol) in dry CH_2Cl_2 (15 mL) was added dropwise. Ice bath was removed and the reaction was stirred at rt for 2 h. The organic phase was washed with H_2O (25 mL), $NaHCO_3$ sat. sol. (25 mL), 1 N HCl (20 mL), brine, dried and the volume was reduced to aprox. 15 mL. The yellow precipitate formed was filtered and washed with light petroleum ether. The crude 4-formyl-*N*-(3-{[4-nitro-3-(trifluoromethyl)phenyl]amino}propyl)benzamide (0.750 g, 1.90 mmol) was dissolved in *t*-BuOH (30 mL), iso-amylene (2.20 mL, 21.0 mmol) and 3 drops of acetic acid were added, followed by a solution of $NaClO_2$ (tech.grade, 0.230 g, 2 mmol c.a.) in H_2O (3 mL). The reaction was vigorously stirred overnight at rt. The organic solvent was evaporated and the residue was dissolved in aqueous oxalic acid sat. sol. (25 mL) and EtOAc (25 mL) mixture. The organic phase was washed with H_2O (25 mL), brine, dried and concentrated to dryness. The resulting solid was crystallized from EtOH to give the desired compound as a yellow powder. Yield 32% for two steps. M.p. 214 – 215 °C (EtOH); ^{1}H-NMR (DMSO-d_6) δ ppm: 1.85 (q, $^3J_{HH}$ = 6.6 Hz; 2H, $CH_2CH_2CH_2$); 3.22–3.42 (m, 4H, $CH_2CH_2CH_2$), 6.81 (dd, $^oJ_{HH}$ = 9.3 Hz; $^mJ_{HH}$ = 2.2 Hz; 1H, C*H*Ar); 7.04 (s, 1H, C*H*Ar); 7.59 (t, $^3J_{HH}$ = 4.9 Hz; 1H, NH); 7.93 - 8.00 (m, 4H, CH_{Ar}); 8.04 (d, $^oJ_{HH}$ = 9.3 Hz; 1H, CH_{Ar}); 8.69 (t, $^3J_{HH}$ = 5.5 Hz; 1H, N*H*); ^{13}C-NMR (DMSO-d_6) δ ppm: 28.1, 37.2, 40.3, 111.2 (m), 111.8 (m), 122.6 (q, $^1J_{CF}$ = 272 Hz, 1C); 125.0 (q, $^2J_{CF}$ = 32.2 Hz, 1C); 127.5,129.3, 129.9, 133.0, 133.5, 138.3, 153.2, 165.8, 166.9; ESI-MS $[M-H]^-$ *m/z* 410.

Doxorubicin 4-[(3-{[4-Nitro-3-trifluoromethyl-phenyl]amino}propyl)carbamoyl]benzoate (DOX-NOP1). To a stirred solution of **NOP-Ac** (550 mg, 1.35 mmol) in dry DMF, KF (155 mg, 2.70 mmol) was added in one portion, and

the reaction was vigorously stirred for 15 min. 14-Bromo/chloro daunorubicin hydrobromide (300 mg, 0.45 mmol) was added and the reaction was stirred at rt for 4 h. Solvent was removed under reduced pressure at 30°C and the resulting mixture was purified by flash chromatography (eluent: gradient from 95/5 to 80/20 CH_2Cl_2/MeOH) to give a dark-red solid. The resulting compound was suspended in dry THF (20 mL), and 2 equivalents of HCl solution in dry dioxane were added. The resulting mixture was stirred for 1 h at rt and then diluted with Et_2O. The resulting precipitate was filtered, washed with Et_2O and dried under vacuum to give the title compound as a red powder. Yield 44%. ^{1}H-NMR (DMSO-*d6*) δ ppm: 1.20 (d, $^3J_{HH}$ = 6.04 Hz, 3H, $^{6'}CH_3$), 1.70 (m, 1H), 1.86 (m, 3H) ($^{2'}CH_2$ + $CH_2CH_2CH_2$), 2.09 (m, 1H), 2.34 (m, 1H, $^8CH_2$), 2.86 (d, $^2J_{HH}$ = 18.7 Hz, 1H), 3.12 (d, $^2J_{HH}$ = 18.7 Hz, 1H, $^{10}CH_2$), 3.28 (m, 4H, $CH_2CH_2CH_2$), 3.63 (br. s, 1H, $^{4'}CHOH$), 3.96 (s, 3H, OCH_3), 4.28 (m, 1H, $^{5'}CH$), 4.94 (m, 1H, ^7CH), 5.30 (s, 1H, $^{1'}CH$), 5.53 (m, 2H, $^{14}CH_2$), 5.83 (br. s, 1H, ^9COH), 6.83 (m, 1H, CH_{Ar}), 7.07 (m, 1H, CH_{Ar}), 7.62 (m, 1H, CH_{Ar}), 7.74 (m, 1H, CONH), 7.86–8.10 (m, 7H, $7CH_{Ar}$), 8.83 (t, 1H, NHAr); ESI-MS $[M+H]^+$: *m/z* 937; ESI-HRMS: $C_{45}H_{44}F_3N_4O_{15}$ *m/z* calcd 937.27498, found 937.27482.

Biological assays

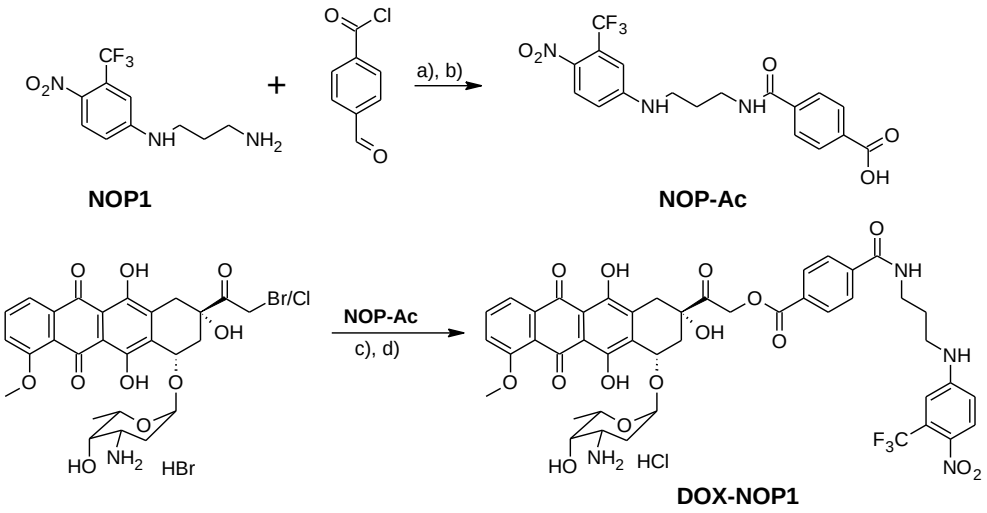
Nitrite release. The amount of extracellular nitrite was measured spectrophotometrically.[3S] Then nitrite production was measured by adding 0.15 ml of cell culture medium to 0.15 ml of Griess reagent (1:1 solution of 0.2% w/v naphthylethylenediamine dihydrochloride, and 2% w/v sulphanilamide in 5% v/v phosphoric acid) in a 96-well plate. After a 10 min incubation at 37 °C in the dark, absorbance was measured at 540 nm with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). A blank was prepared in the absence of cells and its absorbance was subtracted from that measured in the samples. Sodium nitrite was used as a standard to build the calibration curve. Nitrite concentration was expressed as nmoles nitrite/mg cell proteins.

Cytotoxicity. The release of lactate dehydrogenase (LDH) in the extracellular medium, considered an index of DOX cytotoxicity,[4S] was measured spectrophotometrically.[3S] Both intracellular and extracellular LDH were measured; results were first expressed as percentage of extracellular LDH/total (intracellular + extracellular) LDH. The LDH release in the untreated cells was considered as 100%; for each experimental condition, results were then expressed as percentage versus untreated cells.

Immunoblotting. Cells were rinsed with ice-cold lysis buffer (50 mM, Tris, 10 mM EDTA, 1% v/v Triton-X100), supplemented with the protease inhibitor cocktail set III (80 μM aprotinin, 5 mM bestatin, 1.5 mM leupeptin, 1 mM pepstatin; Calbiochem, San Diego, CA), 2 mM phenylmethylsulfonyl fluoride and 1 mM Na_3VO_4, then sonicated and centrifuged at 13000 × g for 10 min at 4 °C. 20 μg protein extracts were subjected to SDS-PAGE and probed with the antibodies for: Pgp (C219, Calbiochem), MRP1 (Abcam, Cambridge, UK), MRP2 (Abcam), MRP3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), MRP4 (Abcam), MRP5 (Santa Cruz Biotechnology Inc.), BCRP (Santa Cruz Biotechnology Inc.), actin (Sigma Chemicals Co.), followed by a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories). The membranes were washed with Tris-buffered saline-Tween 0.1% v/v solution, and the proteins were detected by enhanced chemiluminescence (Bio-Rad Laboratories). To analyze the presence of nitrated proteins, cell extract was subjected to immunoprecipitation using a rabbit polyclonal anti-nitrotyrosine antibody (Millipore, Billerica, MA). Immunoprecipitated proteins were separated by SDS-PAGE and probed with the respective antibodies.

ATPase activity. 1 mg whole cell lysate was immunoprecipitated with antibodies for Pgp, MRP1, MRP3, MRP4, MRP5, BCRP, using the PureProteome protein A and protein G Magnetic Beads (Millipore). The ATPase activity of each immunopurified transporter was measured spectrophotometrically.[5S] Results were expressed as nmoles hydrolyzed phosphate (Pi)/min/mg proteins, according to the titration curve previously prepared.

Statistical analysis. All data in the text and figures are provided as means ± SD. The results were analyzed by a one-way analysis of variance (ANOVA) and Tukey's test, using Statistical Package for Social Science (SPSS) software (IBM SPSS Statistics v.19). $p < 0.05$ was considered significant.



Scheme S1. a) Et_3N, CH_2Cl_2, rt; b) *t*-BuOH, CH_3COOH, $NaClO_2$, 0 °C to rt; c) KF, DMF, rt; d) THF, HCl in dry dioxane.

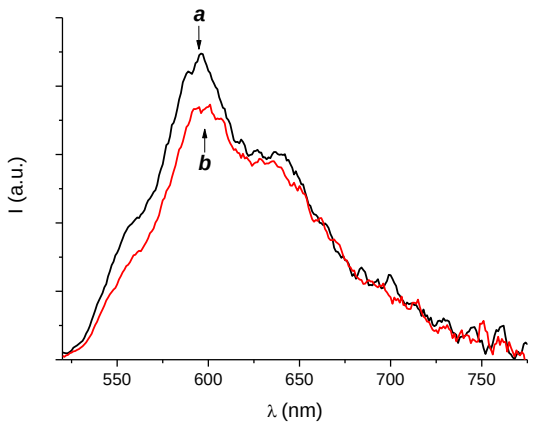


Figure S1. Fluorescence emission spectra of optically matched DMSO solutions of **DOX** (***a***) and **DOX-NOP1** (b). λ_{exc} = 510 nm.

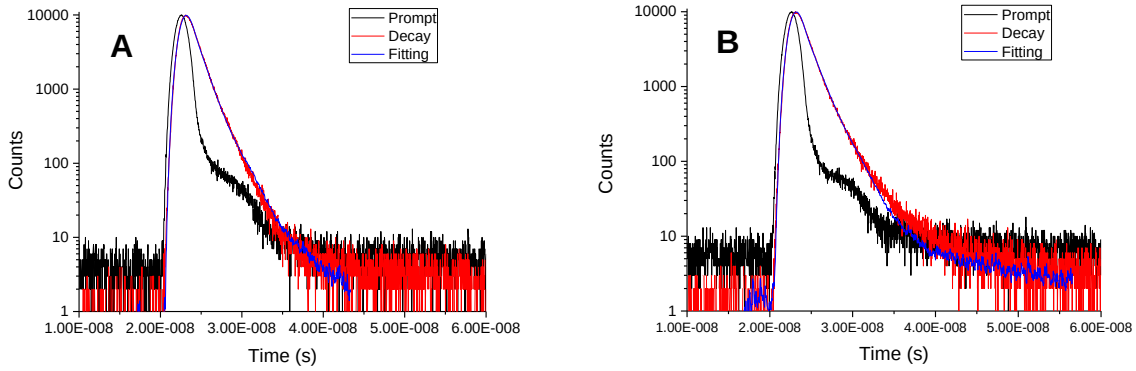


Figure S2. Time-resolved fluorescence decay and related bi-exponential fitting observed for DMSO solutions of **DOX** (**A**) and **DOX-NOP1** (**B**). λ_{ex} = 455 nm; λ_{em} = 595 nm. The kinetic analysis gave lifetimes of 0.035 ns and 1.26 ns for **DOX** and 0.058 ns and 1.44 ns for **DOX-NOP1**, respectively.

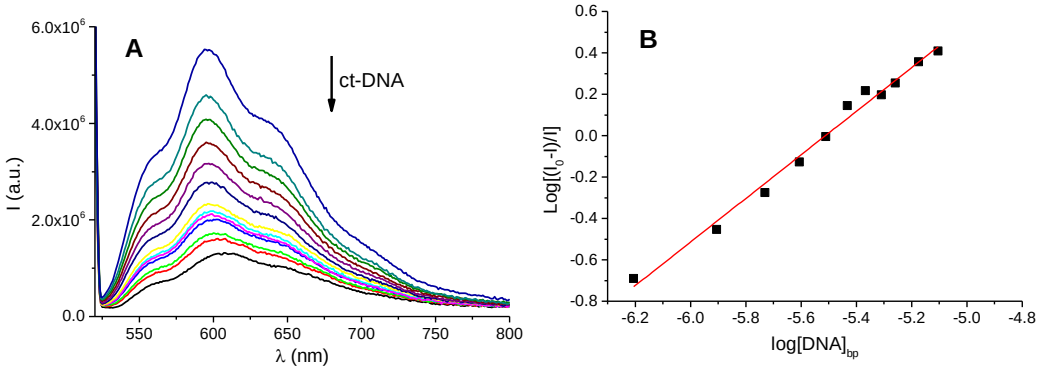


Figure 3S. (A) Fluorescence spectral changes (λ_{ex} = 510 nm) of observed for a PBS (pH 7.4, 10 mM with 3%DMSO) solution of **DOX-NOP1** upon addition of increasing amounts of ct-DNA. [**DOX-NOP1**] = 10 µM. $[\text{ct-DNA}]_{bp}$ from 0 to 20 µM. (B) Linear plot of the fluorescence data (λ_{em} = 590 nm) reported in (A) as a function of the DNA concentration according to eq 1S:[5S]

$$[(I_0\text{-}I)/I] = \log K_b + n \log[\text{DNA}]_{bp} \quad (1S)$$

where I_0 is the initial fluorescence intensity and I the fluorescence intensity at different concentrations of DNA. The binding costant K_b and the number of **DOX-NOP1** molecules per ct-DNA base pairs was calculated from the equation S1 assuming a static quenching regime.

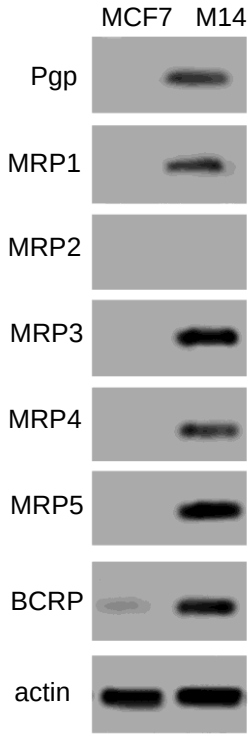


Figure 4S. ABC transporters expression in melanoma cells. The expression of Pgp, MRP1, MRP2, MRP3, MRP4, MRP5, BCRP was measured in human melanoma M14 whole cell lysates by immunoblotting. Actin was used as control of equal protein loading. Human MCF-7 cells were included as cell line with low/undetectable levels of most transporters. The figure is representative of one out of three similar experiments.

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