Supporting Information

A Doxorubicin-NO Releaser Molecular Hybrid Activatable by Green Light to Overcome Resistance in Breast Cancer Cells

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Materials. DXNO-GR was synthesized according to our already previously published procedure.[1S] BSA was purchased by Sigma Aldrich and used as received. All solvent used (from Carlo Erba) were spectrophotometric grade. Cell culture medium and supplements were purchased from Life Technologies (Italy), while sterile plasticwares were from Falcon (Corning, USA).

Instrumentation. UV-Vis spectra absorption and fluorescence emission and excitation 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 monitored at 595 nm, and each solution itself was used to register the prompt at 455 nm. The system allowed the measurement of fluorescence lifetimes from 200 ps. The multiexponential fit of the fluorescence decay was obtained using the following equation:

$$I(t) = \Sigma\alpha_i \exp(^{-t/\tau i})$$

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) under gentle stirring by using a continuum laser with λ_{exc} = 532 nm (ca. 100 mW) having a beam diameter of ca. 1.5 mm. 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.
For the experiments including cells irradiation, it was performed using a green LED lamp (λ_{exc} = 490-540 nm).

Lipophilicity of the drugs. *n*-octanol–water partition coefficients were quantified using a miniaturized shake-flask approach based on OECD 107.[2S] To investigate the analytes in the ionized state occurring in vitro, measurements were performed using 0.1 M phosphate buffer at pH 7.4. Buffer solution was saturated with *n*-octanol (W_o) prior to analysis and vice versa (O_w) by vigorous shaking and rest for 24 h to get spontaneous separation. Stock solutions (1 mL) of DOXO and DXNO-GR (0.5 mg/mL) prepared in O_w or W_o, respectively, were added with the second phase, vortexed for 1 min and then shaken for 2 h (125 rpm) at room temperature (25 ± 3 °C) to reach equilibrium and phase distribution. Following phase separation through centrifugation (2900 x *g*, 5 min), the aqueous or *n*-octanol phases were analyzed separately by UV-vis spectrophotemetry at 490 nm after dilution to obtain an OD around 0.5. Lipophilicity of the compounds (Log $D_{7.4}$) was derived from the difference in the blank corrected OD from the reference stock solution (OD_{ref}) and that in the partitioned samples (OD_{part}) by Log $D_{7.4}$=Log (OD_{ref}/ OD_{part}– 1)

Binding studies with BSA. The protein concentration has been determined spectrophotometrically using the extinction coefficient of 36.500 M^{-1} cm^{-1} at 280 nm. 3 mg of **DXNO-GR** were dissolved in 500 µL of DMSO to obtain a stock solution 6 mM. Titration was carried out by successive addition of this stock solution (from 0 to 80 µM) to a 2.5 mL of a solution of BSA in TRIS-HCl buffer pH 7.4 at 25 °C. The final solutions were left under stirring in the dark for 15 minutes before recording the absorption and emission spectra.

Cells. Human adenocarcinoma MCF-7, triple negative breast cancer MDA-MB-231 cells, and non-tumorigenic breast epithelial MCF-10A cells were purchased from American Type Culture Collection (ATCC, Rockville, USA). MDA-MB-231 and MCF-7 cells were grown in DMEM with Glutamax supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL streptomycin, 100 µg/mL penicillin G and maintained at 37 °C under a humidified atmosphere containing 5% CO_2. MCF-10A cells were cultured in DMEM/F12 medium supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, 100 U/mL streptomycin and 100 µg/mL penicillin G The expression of MDR-related pumps in cells was assessed by flow cytometry using the EFLUXX-ID Green Multidrug Resistance assay kit (Enzo Life Sciences, Italy) and following manufacturer's instructions. This assay allows an evaluation of MDR1 (p-glycoprotein), MRP1/2, BCRP expression on cell membrane calculated as MDR Activity Factor (MAF) by measuring cell retention of the EFLUXX-ID reagent, a substrate of MDR pumps. Briefly, $5x10^5$ cells/sample were incubated for 5 min with verapamil (5 mM), MK-571 (10 mM) and novamicin (50 mM) in order to inhibit MDR1, MRP1/2, BCRP, respectively, before adding the EFLUXX-ID reagent for 30 min at 37 °C. At the end of incubation time, cells were centrifuged, resuspended in Versene and analyzed by the flow cytometer (BD LSR Fortessa X-20), recording EFLUXX-ID reagent fluorescence in the FITC channel. MAF of each MDR pump was calculated as:

$$MAF = (Fi - F_0)/F1 \times 100$$

where Fi is the value of the sample pre-treated with pump inhibitors, F_0 the fluorescence of the sample incubated only with the EFLUXX-ID reagent. When MAF>25 for a certain pump, the cell line is considered MDR-positive.

Dark and photo-toxicity. The cytotoxicity of DXNO-GR and DOX was measured with the MTS assay (Cell-Titer 96 AQueous One Solution Cell Proliferation Assay, Promega Co., USA) according to manufacturer's instructions. 8×10^3 cells/well were seeded in 96-well plates and, after 24 h of growth, treated for 2 h with increasing drug concentrations (1-10 µM). To evaluate cytotoxicity in the absence of irradiation (dark cytotoxicity), at the end of the 2 h of drug exposure, drug treatment was removed, cells washed twice with PBS added with Ca^{2+} and $Mg^{2+}$and then incubated in drug-free medium for further 24 h before assessing cell viability. For phototoxicity experiments, after washing, cells were irradiated with a total dose of 72 J/cm^2 of green light (irradiation time 30 min, power density 40 mW/cm^2) and left for additional 24 h in drug-free medium before measuring cell viability. For the MTS assay the medium was replaced with 100 µL of serum-free medium and 20 µL of the CellTiter 96 reagent. After 1 h, the absorbance at 492 nm was measured with a Multiskan Go (Thermo Fischer Scientific, USA) plate reader and cell viability was expressed as a function of absorbance relative to that of control cells (considered as 100% viability).

Intracellular uptake studies. Cellular uptake of DXNO-GR and DOX was measured by flow cytometry. 6×10^4 cells were grown in 24-well plates for 24 h and incubated for 0.5, 2 and 5 h with 2.5 μM DOX in cell culture medium added with 10% serum. After incubation the cells were washed twice with Versene, detached from the plates with trypsin that was neutralized by the addition of FBS. Cells were centrifuged and resuspended in Versene before measuring DOX fluorescence using a BD LSR Fortessa X-20 flow cytometer. A blue laser at 488 nm was used to excite DOX and its fluorescence was detected at wavelengths longer than 575 nm (PE channel); for each sample 10^5 events were acquired and analyzed using the FACSDiva software. Flow cytometry was used also to measure the capacity of cells to extrude DXNO-GR and DOX during a 5 h incubation. Cells were seeded as described above and pre-treated or not with the MDR1 selective inhibitor Verapamil (50 μM) for 1 h before adding 2.5 μM DXNO-GR or DOX.

Intracellular localization studies. The intracellular localization of DXNO-GR and DOX was evaluated by confocal microscopy. 8 x 10^4 MDA-MB-231 cells were grown in 35 mm imaging dishes (Eppendorf AG, Hamburg, Germany) for 24 h and incubated for 2 or 16 h with 2.5 μM DOX. Fifteen minutes before completing the incubation, cells were stained with Hoechst-33342 (10 μM), BODIPY FL C5-ceramide (7.5 μM), ER-Tracker Green (0.4 μM), LysoTracker Green DND-26 (50 nM) used as probes for nucleus, Golgi apparatus, endoplasmic reticulum and lysosomes, respectively. Cells were then washed twice with HBSS and observed with a Leica SP5 confocal microscope; acquired images were analyzed using ImageJ software.

Intracellular quantification of NO. In order to measure the extent of NO released from DXNO-GR intracellularly after irradiation, we used the probe DAF-FM diacetate (Thermo Fisher Scientific). Cells were seeded as for phototoxicity experiments and incubated for 2 h with 10 or 25 μM DXNO-GR or DOX. At the end of incubation time cells were stained for 20 min with 10 μM of DAF-FM probe and irradiated with green light (72 J/cm^2). After irradiation, cells were rinsed with HBSS and released for 20 min in fresh medium in the dark before reading DAF-FM fluorescence with a Victor3 2030 Multilabel Reader (Perkin Elmer) using the FITC channel. Fluorescence of DAF-FM in treated cells was expressed as fold changes with respect to the fluorescence signal measured in controls and set equal to 1.

MDR1 inhibition by DXNO-GR. The NO capacity to inhibit MDR1 in MDA-MB-231 cells was measured using the EFLUXX-ID kit. Briefly, 0.5 x 10^6 cells were allowed to growth in 50 mm dishes for 24 h, were treated for 2 h with 2.5 μM DOX or DXNO-GR before being exposed or not to green light (72 J/cm^2). Cells were then detached from plates with trypsin, centrifuged and resuspended in medium added with the EFLUXX-ID reagent for 30 min and analysed by flow cytometry as described above.

Statistical analysis. The Primer software for biostatistics (McGraw-Hill, Columbus, OH, USA) was used for statistical analysis of the data. The data are expressed as means ± standard deviations (SD) for at least three independent experiments, carried out in triplicate. The difference between two groups of treatments was evaluated by Student's test while the differences between more than two groups of treatment was evaluated with one-way ANOVA test with the Bonferroni's correction and was considered significant for $p < 0.05$.

References

1S Fraix, A.; Parisi, C.; Failla, M.; Chegaev, K.; Spyrakis, F.; Laz-zarato, L.; Fruttero, R.; Gasco, A.; Sortino, S. NO release regu-ated by doxorubicin as green light-harvesting antenna. *Chem. Commun*., 2020, 56, 6332-6335.

2S Işık, M.; Levorse, D.; Mobley, D. L.; Rhodes, T.; Chodera, J. D. Octanol-water partition coefficient measurements for the SAMPL6 Blind Prediction Challenge, *Journal of Computer-Aided Molecular Design*, 2020, 34, 405–420.

Table S1. MAF values measured by flow cytometry in MCF7 and MDA-MB-231 cells using the EFLUXX-ID Green Multidrug Resistance assay.

Cell line	MDR1	MRP1/2	BCRP
MCF7	*0*	*31*	*22*
MDA-MB-231	*54*	*37*	*24*

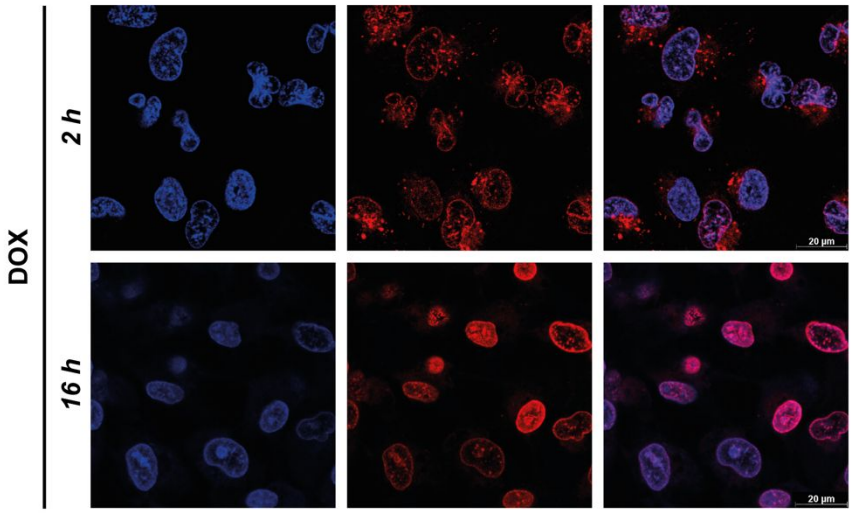

Figure S1. Confocal microscopy images of MDA-MB-231 cells showing DOX (red fluorescence) localization in the nucleus (blue fluorescence, Hoechst-33342 probe) after 2 and 16 h of incubation.

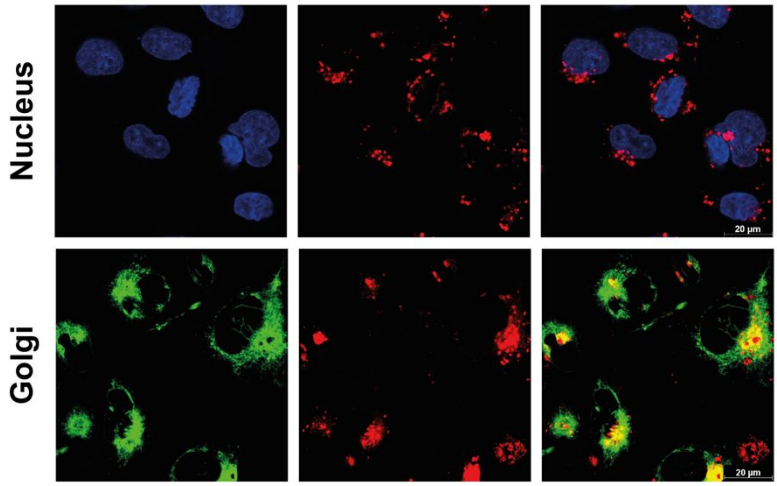

Figure S2. Confocal microscopy images of MDA-MB-231 cells showing the absence of co-localization of DXNO-GR (red fluorescence) and nucleus specific probe Hoechst-33342 (blue fluorescence) after 16 h of incubation. On contrary, at the same incubation time, DXNO-GR clearly localized in the Golgi apparatus.

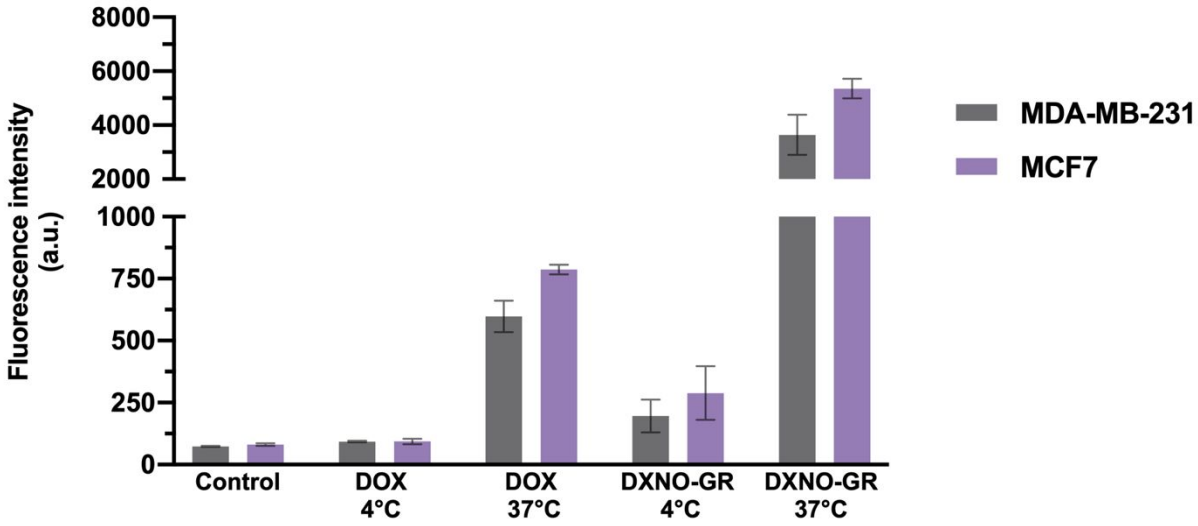

Figure S3. Flow cytometry measurements of intracellular uptake of 2.5 µM DOX and DXNO-GR in MDA-MB-231 and MCF-7 incubated with the drugs for 2 h and maintained at 4 or 37 °C. Data are expressed as mean DOX fluorescence intensity (a.u.) ± SD of two independent experiments, carried out in triplicate.

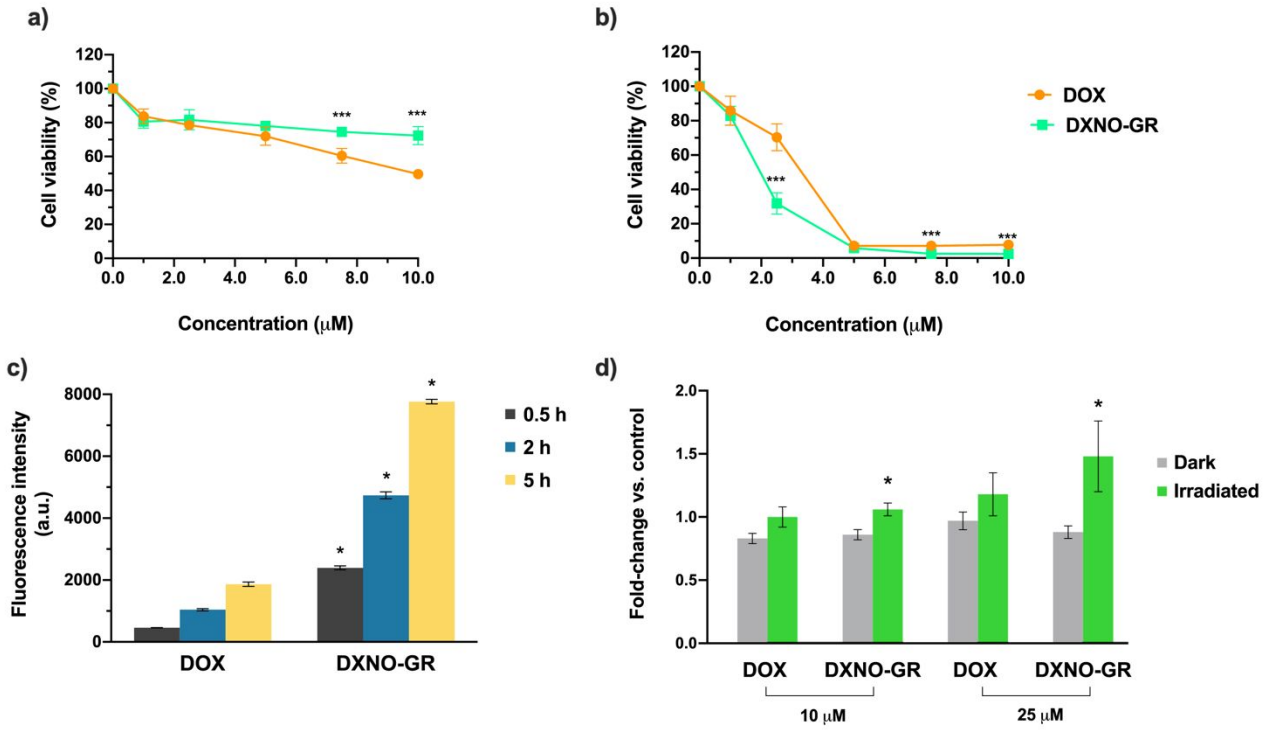

Figure S4. DXNO-GR and DOX behaviors toward non-tumorigenic breast epithelial cells. Dose response curves of MCF-10A cells incubated for 2 h with increasing concentrations of DXNO-GR or DOX in the dark (**a**) or after green light irradiation (**b**). Data are expressed as mean percentage ± SD of at least three independent experiments, carried out in triplicate; ***p<0.001 significantly different from DOX (Student's t Test). **c**) Cellular uptake of 2.5 µM DOX and DXNO-GR measured after different incubation times by flow cytometry. Data are expressed as mean percentage ± SD of at least two independent experiments, carried out in triplicate; *p<0.001 significantly different from DOX (Student's t-Test). **d**) Intracellular levels of NO generated by DXNO-GR or DOX in the absence or in the presence of green light irradiation. NO increment is expressed as increase of DAF-FM probe fluorescence with respect to the fluorescence signal measured in control cells and set equal to 1 (*fold change*). *p<0.001 significantly different from dark sample (Student's t-Test).