Liposomal Nitrooxy-Doxorubicin: One Step over Caelyx in Drug-Resistant Human Cancer Cells

This is the author's manuscript

Original Citation:

Availability:
This version is available http://hdl.handle.net/2318/148171 since 2016-07-14T17:01:21Z

Published version:
DOI:10.1021/mp500257s

Terms of use:
Open Access
Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)
This is an author version of the contribution published on:
Molecular Pharmaceutics 2014, 11, 3068–3079, DOI 10.1021/mp500257s

The definitive version is available at:
[http://pubs.acs.org/journal/mpohbp]
The liposomal nitrooxy-doxorubicin: one step over Caelyx® in drug-resistant human cancer cells.

Isabella Pedrini§,†, Elena Gazzano‡,†, Konstantin Chegaev§, Barbara Rolando§, Alessandro Marengo§, Joanna Kopecka§, Roberta Fruttero§, Dario Ghigo‡, Silvia Arpicco§,*, Chiara Riganti‡,*

§ Department of Drug Science and Technology, University of Torino, via Pietro Giuria 9, 10125, Torino, Italy
‡ Department of Oncology, University of Torino, via Santena 5/bis, 10126, Torino, Italy
† These authors contributed equally to the work.
* These authors contributed equally to the work.

* Corresponding authors: Dr. Silvia Arpicco, Department of Drug Science and Technology, University of Torino, via Pietro Giuria 9, 10125, Torino, Italy; phone: +390116706668; fax: +390116706663; email: silvia.arpicco@unito.it; Dr. Chiara Riganti, Department of Oncology, University of Torino, via Santena 5/bis, 10126, Torino, Italy; phone: +390116705857; fax: +390116705845; email: chiara.riganti@unito.it

ABSTRACT
In this work we prepared and characterized two liposomal formulations of a semisynthetic nitric oxide (NO)-releasing doxorubicin (Dox), called nitrooxy-Dox (NitDox), which we previously demonstrated to be cytotoxic in Dox-resistant human colon cancer cells. Liposomes with 38.2% (Lip A) and 19.1% (Lip B) cholesterol were synthesized: both formulations had similar size and zeta potential values, and caused the same intracellular distribution of free NitDox, but Lip B accumulated and released NitDox more efficiently. In Dox-resistant human colon cancer cells, Lip A and Lip B exhibited a more favorable kinetics of drug uptake and NO release, and a stronger cytotoxicity than Dox and free NitDox. While Caelyx®, one of the liposomal Dox formulations approved for breast and ovary tumors treatment, was ineffective in Dox-resistant breast/ovary cancer cells, Lip B, and to a lesser extent Lip A, still exerted a significant cytotoxicity in these cells. This event was accompanied in parallel by a higher release of NO, which caused nitration of P-glycoprotein (Pgp) and multidrug resistance related protein 1 (MRP1), two transporters involved in Dox efflux, and impaired their pump activity. By doing so, the efflux kinetics of Dox after treatment with Lip B was markedly slowed down and the intracellular accumulation of Dox was increased in breast and ovary drug-resistant cells. We propose these liposomal formulations of NitDox as new tools with a specific indication for tumors overexpressing Pgp and MRP1.

KEYWORDS: doxorubicin, nitric oxide, liposomes, P-glycoprotein

ABBREVIATIONS: ABC, ATP binding cassette; Pgp, P-glycoprotein; MRP, multidrug resistance related protein; BCRP, breast cancer resistance protein; NO, nitric oxide; Dox, doxorubicin; NitDox, nitrooxy-Dox; EPR, enhanced permeability and retention; FBS, fetal bovine serum; Chol, cholesterol; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE-
INTRODUCTION

The overexpression of ATP binding cassette (ABC) transporters, such as P-glycoprotein (Pgp/ABCB1), multidrug resistance related proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2), on the tumor cell surface limits the accumulation and cytotoxicity of chemotherapeutic drugs, and of new targeted-therapy molecules like tyrosine kinase inhibitors. Owing to the overlapping spectrum of drugs effluxed by each transporter, the presence of one or more ABC transporters confers to tumor cells the multiple cross-resistant phenotype known as multidrug resistance (MDR). Despite promising results in vitro, pharmacological inhibitors of ABC transporters have failed in vivo for their poor specificity and high toxicity.

We recently found that nitric oxide (NO) donors inhibit the ABC transporter-mediated efflux, by nitrating critical tyrosines for the catalytic activity of these proteins. Since doxorubicin (Dox) is one of the chemotherapeutic drugs with the widest spectrum of antitumor activities, but it is ineffective in tumor overexpressing Pgp, MRP1, MRP2, MRP3, BCRP, we produced semisynthetic Doxs conjugated with NO-releasing groups. Owing to the NO-releasing property, these Doxs were less effluxed, more accumulated and more cytotoxic in drug-resistant cells. One of them, nitrooxy-Dox (NitDox), revealed also pharmacodynamic properties completely different from parental Dox: NitDox had a different kinetics of uptake and a predominant
cytosolic and mitochondrial localization, impaired the mitochondrial aerobic metabolism, increased the generation of reactive oxygen and nitrogen species, induced a caspase 9-mediated cell death in both drug-sensitive and drug-resistant cells\textsuperscript{10}.

An increasing body of evidences demonstrated that chemotherapeutic drugs encapsulated in liposomal-based formulations, alone or combined with chemosensitizing agents or specific shRNA targeting ABC transporters, are more effective than free drugs in resistant tumors\textsuperscript{11-13}. The molecular basis of such efficacy has not been fully understood yet. The proposed mechanisms include: slower drug release from liposomes\textsuperscript{14,15}, lower induction of Pgp\textsuperscript{16}, different intracellular drug distribution\textsuperscript{15}, changes in the lipid environment wherein Pgp optimally works\textsuperscript{17}, inhibition of Pgp catalytic activity exerted by specific components of liposomal shell\textsuperscript{18}.

A second advantage of liposomal formulations is the greater drug delivery within the tumor core owing to the enhanced permeability and retention (EPR) effect\textsuperscript{19}, with consequent decrease of side effects in non-transformed tissues.

The PEGylated liposomal formulation of Dox, known as Doxyl\textsuperscript{®} or Caelyx\textsuperscript{®}, has been approved for the treatment of metastatic breast and ovary cancers\textsuperscript{20}. Besides being more effective than free Dox, liposomal formulations exhibit reduced cardiotoxicity than free Dox\textsuperscript{21}.

In the present work we produced two liposomal formulations of NitDox and verified that they maintain some peculiar features of free NitDox, such as the typical cytosolic distribution, the fast kinetics of uptake, the high cytotoxicity and ability to release NO in resistant cells. Next, we compared our liposomal formulations of NitDox with Caelyx\textsuperscript{®} in human breast and ovary cancer cells with different degrees of Dox-resistance, to assess whether the new formulations exert in resistant cells greater therapeutic benefits than the clinically approved formulation.
**EXPERIMENTAL SECTION**

**Chemicals.** The phospholipids were provided by Avanti Polar-Lipids (distributed by Spectra 2000, Rome, Italy). Cholesterol, Dox and the other chemicals were obtained from Sigma Chemical Co. (St. Louis Mo, USA). The solvents used were of analytical grade, purchased from Carlo Erba Reagenti (Milan, Italy). Fetal bovine serum (FBS) and culture media were supplied by Invitrogen Life Technologies (Carlsbad, CA). The 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. The synthesis of NitDox was performed as described previously\(^9\).

**Preparation of liposomes.** NitDox was encapsulated in two different liposome formulations characterized by a different amount of cholesterol (Chol) and prepared by the thin lipid film hydration and extrusion method. Briefly, two chloroform solutions of 1,2-distearoyl-\(sn\)-glycero-3-phosphocholine (DSPC), Chol and 1,2-distearoyl-\(sn\)-glycero-3-phosphoethanolamine-N-\[amino(polyethylene glycol)-2000\] (DSPE-PEG, ammonium salt), having molar ratios 56.5:38.2:5.3 (Lip A) and 75.6:19.1:5.3 (Lip B) respectively and containing NitDox (11% moles of drug/moles of lipids ratio), were evaporated. The resulting lipid film was dried under vacuum overnight, then hydrated with a 20 mM HEPES buffer (pH 7.4): this suspension was vortexed for 10 min and bath sonicated. The formulations were then extruded (Extruder, Lipex, Vancouver, Canada) passing the suspension 20 times under nitrogen through a 400 and a 200 nm polycarbonate membrane (Costar, Corning Incorporated, NY), at a set temperature of 5°C above the phase transition temperature of the lipid mixture. The liposomal preparations were purified
from non-encapsulated NitDox through chromatography on Sepharose CL-4B columns, eluted with HEPES buffer at room temperature. The liposomes were stored at 4°C.

**Liposome characterization.** The mean particle size and polydispersity index of the liposomes were determined at 20°C by quasi-elastic light scattering (QELS) using a nanosizer Coulter® N4MD (Coulter Electronics, Inc., Hialeah, FL). The selected angle was 90° and the measurement was performed after dilution of the liposome suspensions in MilliQ® water. Each measurement was carried out in triplicate. The surface charge of liposomes was evaluated by zeta potential measurements after diluting the suspension in 10 mM KCl, using a Zetasizer (Zeta Potential Analyzer Ver. 2.17, Brookhaven Inst. Corp., Holtsville, NY).

The phospholipid phosphorous was measured in each liposome preparation by phosphate assay after liposome disruption in perchloric acid\(^{22}\).

The amount of NitDox incorporated was determined by reverse phase-high pressure liquid chromatography (RP-HPLC). The liposomal formulation was diluted with acetonitrile/HCOOH (v/v 100:0.1), sonicated, vortexed, centrifuged for 10 min at 2,150 x g; then the clear supernatant was filtered through 0.45 µm filters (Alltech). The 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 in 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 flow-rate of 1.0 ml/min. The injection volume was 20 µl. The column effluent was monitored at 234 nm and 480 nm, referenced against a 700 nm wavelength. By RP-
HPLC procedure, we separated and quantified NitDox and eventual degradation products (Dox, aglycone of NitDox). The quantification of NitDox and Dox was done using a calibration curve obtained with standard solutions chromatographed in the same experimental conditions, with a concentration range of 1–200 μM ($r^2 > 0.99$).

**Differential Scanning Calorimetry (DSC).** The DSC analysis was performed on hydrated samples using a differential scanning calorimeter (DSC 7, PerkinElmer, Waltham, MA) equipped with an instrument controller Tac7/DX (PerkinElmer). The liposomes composed by DSPC alone were prepared with the above described extrusion technique. About 20 mg of accurately weighted samples (unloaded liposomes, NitDox, NitDox-containing liposomes with different amounts of cholesterol) were introduced into a standard aluminum sample pan and analyzed. The DSC runs were performed from 20°C to 85°C, at a rate of 2°C/min under a constant nitrogen stream (40 ml/min). The main transition temperature (Tm) was determined as the onset temperature of the highest peak. The enthalpy values ($\Delta H_m$) were calculated from the area under the main transition peak. The calibration was achieved using indium (Tm = 156.83°C) and n-decan (Tm=−29.6°C).

**Chemical stability of NitDox in phosphate buffer (pH 7.4) and in human serum.** For the stability study in phosphate-buffered saline (PBS), a solution of NitDox (10 mM in DMSO/methanol) or liposomal NitDoxs (Lip A or Lip B) was added to PBS pre-heated at 37°C; the final concentration of the compound was 50 μM. The solution was incubated at 37 ± 0.5°C, and at appropriate time intervals it was analyzed by RP-HPLC as described above. For the stability study in human serum, the solutions of NitDox or liposomal NitDoxs were added to human serum (sterile filtered from human male AB plasma, Sigma-Aldrich) pre-heated 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 an aliquot of 300 µl 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 2,150 x g. The clear supernatant was filtered through 0.45 µm PTFE filters (Alltech) and analyzed by RP-HPLC as described above. All experiments were performed at least in triplicate.

**Physical stability of liposomes.** The liposomal preparations were analyzed for physical stability in the storage conditions, evaluating – at different interval times – diameter, zeta potential and drug leakage at 4°C. The drug leakage was determined on 200 µl of liposomes purified by chromatography on Sepharose CL-4B columns, eluted with HEPES buffer and re-analyzed for drug and phospholipid content as described above. The change in drug content was interpreted as an index of liposome instability.

To evaluate the NitDox release in fetal calf serum (FCS), the formulations were diluted 1:2.5 with FCS and incubated at 37°C for different periods of time. The NitDox-loaded liposomes were separated by chromatography from the leaked drug as previously described. The drug and lipid contents were measured in the collected liposomal fractions and compared with initial values. For comparison, a drug leakage study was also performed in HEPES buffer at 37°C.

**Cells.** Human colon cancer Dox-sensitive HT29 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium. A sub-population of Dox-resistant HT29 cells, namely HT29-dx, was created as previously reported and subsequently cultured in RPMI 1640 medium containing 200 nM Dox. Rat cardiomyocytes H9c2 cells were grown in DMEM medium. Human breast cancer Dox-sensitive MCF7 cells (ATCC) were cultured in DMEM/Ham F12 medium; a sub-population of Dox-resistant MCF7 cells, namely MCF7-dx, was created by culturing parental cells for 40 passages with increasing concentrations of Dox. The cells were subsequently maintained in
DMEM/Ham F12 medium containing 50 pM Dox. Human breast cancer MDA-MB-231 cells (from ATCC) were grown in RPMI 1640 medium and were constitutively Dox-resistant\textsuperscript{23}. Primary Dox-resistant HP06 cells, derived from the peritoneal metastasis of a female patient with an invasive breast cancer\textsuperscript{24}, were a gift of Prof. Anna Sapino (Department of Medical Science, University of Turin, Italy): they were maintained in RPMI 1640 medium and used at passages 2-4. The use of HP06 was approved by the Bioethics Committee of the University of Turin, Italy. Human ovary cancer Dox-sensitive A2780 cells were purchased from ECACC (Salisbury, UK) and cultured in DMEM medium; a sub-population of Dox-resistant A2780 cells, namely A2780-dx, was created by culturing parental cells for 20 passages with increasing concentrations of Dox. Cells were then maintained in DMEM medium containing 50 nM Dox. HT29-dx, MCF7-dx and A2780-dx were used as models of acquired resistance to Dox; MDA-MB-231 and HP06 cells were used as models of intrinsic resistance to Dox. The culture media were supplemented with 10% FBS, 1% penicillin-streptomycin and 1% L-glutamine. The cell cultures were maintained in a humidified atmosphere at 37°C and 5% CO\textsubscript{2}.

**Intracellular Dox distribution and accumulation.** To detect intracellular Dox distribution, 5 \( \times \) 10\textsuperscript{5} cells were grown on sterile glass coverslips, and incubated with 5 \( \mu \)M Dox, free NitDox, Lip A, Lip B, as indicated in the Results section. At the end of the incubation period, the cells were rinsed with PBS, fixed with 4% w/v paraformaldehyde for 15 min, washed three times with PBS and once with water. The slides were mounted with 4 \( \mu \)l of Gel Mount Aqueous Mounting and examined. The image acquisition was performed with a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) with 60 \( \times \) oil immersion objective and 10 \( \times \) ocular lens. In a parallel set of experiments, after fixation the cells were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, diluted 1:10,000) for 3 min
at room temperature in the dark. The cells were washed three times with PBS and once with water, then the slides were mounted and examined with a Leica DC100 fluorescence microscope, with 63 × oil immersion objective and 10 × ocular lens. For each experimental point, a minimum of five microscopic fields were examined.

The intracellular accumulation of Dox was detected spectrofluorimetrically, as described. Fluorescence was converted into nmol Dox/mg cell proteins using a calibration curve prepared previously.

**Lactate dehydrogenase (LDH) release.** To verify the cytotoxic effects of Dox, the activity of LDH was measured in the extracellular medium and in the cell lysate, as reported previously. The reaction was followed for 6 min, measuring the absorbance at 340 nm with Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT), and was linear throughout the time of measurement. Both intracellular and extracellular enzyme activity was expressed in μmol NADH oxidized/min/dish, then extracellular LDH activity was calculated as percentage of the total LDH activity in the dish.

**Nitrite synthesis.** The cells were incubated in fresh medium under the experimental conditions indicated in Results. The nitrite production was measured by the Griess method, reading the absorbance at 540 nm with a Packard EL340 microplate reader (Bio-Tek Instruments). A blank was prepared for each experiment in the absence of cells, and its absorbance was subtracted from the one obtained in the presence of cells. The nitrite concentration was expressed as nmol/mg cell proteins.

**Western blot analysis.** The cells were rinsed with 0.5 ml boiling lysis buffer (10 mM Tris, 100 mM NaCl, 20 mM KH2PO4, 30 mM EDTA, 1 mM EGTA, 250 mM sucrose; pH 7.5). After sonication, 1 mM Na3VO4, 1 mM NaF, 10 mM dithiothreitol and the inhibitor cocktail set III
[100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 mM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin; Calbiochem, San Diego, CA] were added. The cell lysates were centrifuged at 13,000 x g for 15 min. 50 µg cell proteins were separated by SDS-PAGE and probed with anti-Pgp/ABCB1 (C219, Calbiochem), anti-MRP1/ABCC1 (Abcam, Cambridge, UK), anti-MRP2/ABCC2 (Abcam), anti-MRP3/ABCC3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-BCRP/ABCG2 (Santa Cruz Biotechnology) and anti-pancadherin (Santa Cruz Biotechnology, used as control of equal loading) antibodies, followed by the peroxidase-conjugated secondary antibody (Bio-Rad). The membranes were washed with Tris-buffered saline (TBS)-Tween 0.1% v/v, and the proteins were detected by enhanced chemiluminescence (Bio-Rad).

To detect Pgp, MRP1, MRP3, BCRP nitrated on tyrosine, the whole cell lysate was immunoprecipitated with an anti-nitrotyrosine antibody (Millipore, Bedford, MA), using the PureProteome protein A/protein G Magnetic Beads (Millipore). The immunoprecipitated proteins were separated by SDS-PAGE and probed with anti-Pgp/ABCB1, anti-MRP1/ABCC1, anti-MRP3/ABCC3, anti-BCRP/ABCG2 antibodies, as previously described.

**ATPase assay.** The assay was performed on Pgp-enriched membrane vesicles, immunoprecipitated MRP1 or BCRP proteins, according to\(^\text{18}\). The absorbance of the phosphate hydrolyzed from ATP was measured at 620 nm, using a Packard EL340 microplate reader (BioTek Instruments). The absorbance was converted into µmol hydrolyzed phosphate/min/mg proteins, according to the titration curve previously prepared.

**Kinetic parameters of Dox efflux.** The MCF7-dx cells were incubated for 20 min with increasing (0-50 µM) concentrations of Dox, NitDox, Lip A or Lip B, then washed and analyzed for the intracellular concentration of Dox. A second series of dishes, after incubation in the same
experimental conditions, were left for further 10 min at 37°C, then washed and tested for the intracellular Dox content. The difference of Dox concentration between the two series, expressed as nmol Dox extruded/min/mg cell proteins was plotted versus the initial drug concentrations. The values were fitted to Michaelis-Menten equation to calculate Vmax and Km, using the Enzfitter software (Biosoft Corporation, Cambridge, United Kingdom).

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

**RESULTS**

**Preparation and characterization of liposomal NitDoxs**

The NitDox liposomal formulations were prepared by extruding multilamellar liposomes composed of DSPC, DSPE-PEG and two different amounts of Chol (Lip A and Lip B). Lip A was composed of a phospholipid mixture similar to that of Caelyx®: Chol was 38.2% of the total lipids in Lip A and 19.1% in Lip B. The physico-chemical characteristics of the different formulations are summarized in Table 1. Hydration of the drug-lipid film, followed by 20 cycles of extrusion through 400 and 200 nm polycarbonate filters, was a feasible method to obtain homogenous small unilamellar vesicles, having particle size around 210 nm and a low polydispersity index (<0.12). The liposomes showed a similar zeta potential value of about –7 mV (Table 1). On the contrary, the efficiency of drug incorporation in the vesicles was affected by the concentration of Chol in the phospholipid mixtures, since we found that the amount of encapsulated NitDox increased with decreasing Chol. As reported in Table 1, Lip A showed an entrapment efficiency value around 80% Different formulations of liposomes were tested in
order to increase the NitDox loading (data not shown): among them, Lip B showed the greatest incorporation efficiency (95%).

**Table 1**

Characteristics of NitDox-containing liposomes (means, $n=3$).

<table>
<thead>
<tr>
<th>Phospholipid composition</th>
<th>Mean particle size (nm ± SD)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV ± SD)</th>
<th>Entrapment efficiency (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC/Chol/DSPE-PEG (56.5:38.2:5.3)</td>
<td>208 ± 6</td>
<td>0.117</td>
<td>-7.39 ± 1.1</td>
<td>80.8 ± 2.6</td>
</tr>
<tr>
<td><strong>Lip A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPC/Chol/DSPE-PEG (75.6:19.1:5.3)</td>
<td>211 ± 5</td>
<td>0.119</td>
<td>-7.45 ± 1.1</td>
<td>95.7± 2</td>
</tr>
<tr>
<td><strong>Lip B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Ratio between drug/lipid molar ratio after extrusion and drug/lipid molar ratio after purification.

**DSC analysis**

The DSC was performed to investigate the thermal changes caused by the incorporation of NitDox and the different amounts of Chol in the phospholipid bilayer. The results are reported in Table 2. Pure DSPC liposomes showed a main transition peak at 52.19 °C ($T_{onset}$) with a $\Delta H_m$ of 34.98 J/g corresponding to the gel-to-liquid-crystalline transition. The incorporation of Chol in DSPC liposomes dramatically altered the calorimetric profile: the main transition was shifted to a lower temperature ($T_{onset}$ 42.40°C for liposomes composed of 19.1% Chol) or abolished with increasing Chol to 38.2%. This behavior could be explained by a perturbation of phase transition, caused by the strong interaction of Chol with the liposomes’ bilayer.
The addition of NitDox at DSPC/Chol liposomes increased the Tm and the ΔHm in comparison to the formulations without the drug, proving the incorporation of NitDox in the bilayer.

**Table 2.**

Thermodynamic data determined by DSC of different liposomal formulations.

<table>
<thead>
<tr>
<th></th>
<th>Onset (°C)</th>
<th>ΔH (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NitDox</td>
<td>73.13</td>
<td>341.86</td>
</tr>
<tr>
<td>DSPC</td>
<td>52.19</td>
<td>34.98</td>
</tr>
<tr>
<td>DSPC/Chol (19.1%)</td>
<td>42.40</td>
<td>10.93</td>
</tr>
<tr>
<td>DSPC/Chol (38.2%)</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Lip B</td>
<td>48.48</td>
<td>25.27</td>
</tr>
<tr>
<td>Lip A</td>
<td>41.36</td>
<td>15.31</td>
</tr>
</tbody>
</table>

**Chemical stability of NitDox and liposomal NitDox**

The stability of free NitDox and liposomal NitDox was studied in PBS at pH 7.4 and in human serum at 37°C to evaluate the protective effect of Lip A and Lip B. In PBS NitDox was quite stable, maintaining the 70% of initial concentration after 24 h of incubation; in these conditions aglicone was the main degradation product. As expected, the Lip A and Lip B formulations increased the stability of NitDox improving its retention up to 90% after 24 h. In human serum NitDox was hydrolyzed to Dox and substructures containing nitrooxy functions with a half-life of 16 h; by contrast, when incorporated in Lip A and Lip B, NitDox had a half-life of 25 and 19 h respectively. These results clearly showed that the liposomal formulation effectively protects NitDox from the action of human serum esterase.

**Physical stability of liposomal NitDox**
The stability of liposomes was evaluated in storage conditions (HEPES buffer at 4°C), in FCS and HEPES buffer at 37°C. The drug leakage was determined by removing aliquots of liposomes at different time intervals and re-evaluating drug and phospholipid content after purification. The formulations containing the two different amounts of Chol were stable in the storage conditions: after 28 days they still conserved 85% of the initial drug content. Moreover the mean diameter of liposomes stored at 4°C was evaluated: the results showed that the liposomal preparations were stable for at least 28 days. Over this period no appreciable size change (<10% for all the preparations) and no precipitation or liposomes aggregation were observed. Similarly, the zeta potential remained unchanged for the same time period.

The release profile of NitDox from liposomes was also evaluated in FCS and HEPES buffer at 37°C. As shown in Figure 1, the drug leakage was higher in both FCS and HEPES buffer for liposomes containing the lower Chol amount (Lip B); Lip A released 50% of the drug after 48 h in buffer and after 24 h in serum, while Lip B released 50% of the drug after 24 h in buffer and after 16 h in serum. These results clearly showed that a decrease of Chol causes increased drug leakage from liposomes, as previously reported. At 72 h in serum the amount of drug present in both formulations was no more detectable by HPLC analysis (not shown).
Figure 1. Release of NitDox from liposomes in HEPES buffer and in serum at 37°C. The results are expressed as percentage of the initial drug remaining in liposomes, at different time periods, and are presented as mean ± SD (n = 3).

Liposomal NitDoxs are more effective than free NitDox and Dox in accumulating and exerting toxic effects in drug-resistant colon cancer cells

We previously observed that Dox has a nuclear localization, whereas NitDox has a cytosolic localization in Dox-sensitive human HT29 colon cancer cells. By analyzing the intracellular distribution of liposomal NitDoxs, we found the same cytosolic localization in HT29 cells with both formulations (Figure 2A and Supplementary Figure 1); by contrast, free Dox was mainly accumulated in the nucleus, as suggested by the overlap between the fluorescence of Dox and the fluorescence of the nuclear dye DAPI (Supplementary Figure 1). The free NitDox was detected inside the cells earlier than Lip A and Lip B (Figure 2A). HT29 and HT29-dx cells showed a different kinetics of intracellular Dox accumulation as a function of the formulation: the free NitDox was significantly higher than Dox within HT29 cells after 1 h, reached the maximum concentration at 3 h and maintained the same intracellular amount of Dox at 24 h; by contrast, the liposomal NitDoxs content increased progressively, reaching the highest concentration at 24
h, when it was significantly higher than Dox and free NitDox (Figure 2B). In HT29-dx cells, the concentration of intracellular Dox was very low at each time, as expected (Figure 2B); the free NitDox reached the highest concentration at 3 h and then progressively decreased, although it remained significantly higher than Dox at 24 h. Notably, the intracellular accumulation of liposomal NitDoxs followed the same kinetics in HT29 and HT29-dx cells, reaching similar concentrations at 24 h in both cell populations (Figure 2B).

Figure 2. Liposomal NitDoxs have the same intracellular localization of free NitDox, but are more accumulated in drug-resistant cells
A. HT29 cells were incubated with 5 µM Dox, nitrooxy-Dox (NitDox), liposomal formulations of Nitdox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively). At different time periods, the cells were washed, fixed and analyzed for the intracellular localization of Dox by confocal microscopy. The samples were analyzed by Nomarski differential interference contrast (DIC) optics and by fluorescence (fluo) confocal microscope. Magnification: 60 x objective (1.4 numerical aperture); 10 x ocular lens. Bar: 10 µm. The images are representative of three experiments with similar results. B. HT29 cells and Dox-resistant HT29-dx cells were incubated as described in A. At different time points, the cells were lysed and the intracellular amount of Dox was quantified fluorimetrically. Data are presented as mean ± SD (n = 3). For both panels, NitDox versus Dox: * p < 0.001; Lip A/Lip B versus Dox: ° p < 0.002; Lip A/Lip B versus NitDox: ° p < 0.002.

To evaluate the long term cytotoxicity and NO production, HT29 and HT29-dx cells were incubated with each formulation up to 72 h: Dox produced time-dependent cytotoxicity (Figure 3A) and increased the NO synthesis (Figure 3B) in HT29 cells, but not in HT29-dx cells (Figure 3A-3B). Free NitDox was significantly more cytotoxic and elicited a greater release of NO than Dox in both drug-sensitive and drug-resistant cells. Again, the liposomal NitDoxs exerted stronger effects than Dox and NitDox, without exhibiting significant differences between HT29 and HT29-dx cells (Figure 3A-B). Interestingly, Lip B was more cytotoxic (Figure 3A) and released more NO (Figure 3B) than Lip A in resistant cells.

These data suggest that liposomal NitDoxs were more effective than Dox and free NitDox in drug-resistant cells. Dose-dependence experiments confirmed this suggestion: both liposomal NitDoxs began to exert cytotoxic effects at 1 µM in HT29 cells and at 0.5 µM in HT29-dx cells, a concentration at which neither Dox nor free NitDox induced cell damages (Supplementary Figure 2A). At 5 µM (i.e. the concentration at which Dox was cytotoxic in drug-sensitive cells but not in drug-resistant ones, and NitDox was cytotoxic in both cell populations), the liposomal NitDoxs were significantly more cytotoxic than Dox and free NitDox in HT29-dx cells (Supplementary Figure 2A). In parallel, the liposomal NitDoxs released more NO than Dox and free NitDox in both HT29 and HT29-dx cells (Supplementary Figure 2B).
Given the higher cytotoxicity of liposomal NitDoxs, we measured in parallel their cytotoxic effects on H9c2 cells, since the cardiomyocytes are the most sensitive cells to Dox-induced damages. Interestingly, the liposomal NitDoxs, as well as free NitDox, produced a lower release of extracellular LDH than Dox (Supplementary Figure 3A), together with a greater increase of NO levels (Supplementary Figure 3B). The time-dependent measurement of the intracellular drug content showed that liposomal NitDoxs were less accumulated than Dox and free NitDox within H9c2 cells (Supplementary Figure 3C).

Figure 3. Liposomal NitDoxs are more cytotoxic and release more NO than free NitDox in drug-resistant cells

A-B. The drug-sensitive HT29 cells and drug-resistant HT29-dx cells were grown in fresh medium (Ctrl) or medium containing 5 μM Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively) for 24, 48, 72 h. At each time point, the cell supernatants were collected, and checked spectrophotometrically for the release of LDH (panel A), taken as an index of drug toxicity, and for the amount of nitrite (panel B), taken as an index of NO levels. Data are presented as mean ± SD (n = 3). For all time points in panels A-B: versus untreated (Ctrl) cells: * p < 0.001; Lip A/Lip B/NitDox versus Dox: ° p < 0.02; Lip A/Lip B versus NitDox: ° p < 0.05.
Liposomal NitDoxs are more effective than the clinically used liposomal Dox against breast and ovary drug-resistant cancer cells

The liposomal formulations of Dox, e.g. Doxyl® or Caelyx®, are widely used in the clinical treatment of breast and ovary tumors. We therefore compared the effects of our liposomal formulations of NitDox with those of Caelyx®, in a panel of breast and ovary cancer cells, showing different expression of the ABC transporters – i.e. Pgp, MRP1-3, BCRP – involved in the efflux of Dox (Figure 4A). Dox, free NitDox, liposomal NitDoxs and Caelyx® did not show significant differences of cytotoxicity in drug-sensitive cells, such as breast MCF7 cells and ovary A2780 cells (Figure 4B). The free NitDox was cytotoxic in resistant MCF7-dx and A2780-dx cells; by contrast, it was devoid of effects in constitutively resistant MDA-MB-231 and HP06 cells, which were insensitive to Dox’s cytotoxicity (Figure 4B). Amongst the liposomal formulations, Caelyx® did not show any significant increase of toxicity versus Dox in drug-resistant cells, Lip A was cytotoxic against MCF7-dx cells but not against the other drug-resistant cell lines, whereas Lip B was the only formulation significantly cytotoxic in all the drug-resistant cell lines that we tested (Figure 4B).
Figure 4. Liposomal NitDoxs are more cytotoxic than Caelyx® in breast and ovary resistant cancer cells

A. Dox-sensitive (MCF7) and Dox-resistant (MCF7-dx, MDA-MB-231, HP06) breast cancer cells, Dox-sensitive (A2780) and Dox-resistant (A2780-dx) ovary cancer cells were analyzed for the expression of Pgp, MRP1, MRP2, MRP3, BCRP by Western blotting. The expression of total cadherins, checked with a pan-cadherin antibody, was used as a control of equal protein loading. The figure shown here is representative of two independent experiments with similar results. B. The same cell populations of panel A were grown for 24 h in fresh medium (Ctrl) or medium containing 5 µM Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively), Caelyx®. Then the cell supernatants were collected and the release of extracellular LDH was measured spectrophotometrically. Data are presented as mean ± SD (n = 4). Versus untreated (Ctrl) cells: * p < 0.05; Lip A/Lip B versus Dox: ° p < 0.02; Lip A/Lip B versus Caelyx®: ♦ p < 0.05.

As far as the NO amount was concerned, Caelyx® showed the same efficacy of Dox: it increased NO levels in drug-sensitive but not in drug-resistant cell lines (Figure 5A). Again, the free NitDox induced a significant increase of NO in cell lines with acquired drug-resistance (MCF7-dx and A2780-dx cells), but not in cells with a constitutive and strong drug-resistant
phenotype (MDA-MB-231 and HP06 cells). Lip A increased the NO amount in all but HP06 cells; only Lip B induced a significant increase of NO levels in all the cell lines (Figure 5A).

In keeping with these data, Dox did not elicit any tyrosine nitration on Pgp isolated from MCF7-dx cells, and Caelyx® induced a hardly detectable nitration. The free NitDox, and – to a greater extent – Lip A and Lip B induced a stronger nitration of the protein (Figure 5B). Since the nitration of Pgp is usually associated with a reduced catalytic activity, we next measured the ATPase activity of Pgp-enriched membrane vesicles isolated from MCF7-dx cells, in basal conditions (Figure 5C) and after stimulation with verapamil, to detect the maximal rate of ATP hydrolysis (Figure 5D). Under basal conditions, the ATPase activity of Pgp-enriched membranes was not significantly changed by Dox, showed a small decrease with Caelyx®, but a significant decrease was observed with free NitDox and in particular with the liposomal NitDoxs (Figure 5C). The drugs reduced the maximal catalytic activity of Pgp following this rank order: Lip B > Lip A > free NitDox > Caelyx®; Dox was unable to reduce the verapamil-stimulated ATPase activity (Figure 5D).
Figure 5. Liposomal NitDoxs produce a stronger nitration of Pgp and markedly reduce Pgp ATPase activity

A. Dox-sensitive (MCF7) and Dox-resistant (MCF7-dx, MDA-MB-231, HP06) breast cancer cells, Dox-sensitive (A2780) and Dox-resistant (A2780-dx) ovary cancer cells were grown in fresh medium (Ctrl) or medium containing 5 µM Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively), Caelyx® for 24 h. The nitrite levels were measured in cell supernatants spectrophotometrically. Data are
presented as mean ± SD (n = 4). Versus untreated (Ctrl) cells: * p < 0.05; Lip A/Lip B versus Dox: † p < 0.01; Lip A/Lip B versus Caelyx®: ° p < 0.02. B. MCF7-dx cells were incubated as reported in A, then lysed and subjected to the following investigations. To detect nitrated Pgp (nitro-Pgp), the samples were immunoprecipitated with an anti-nitrotyrosine antibody, then probed with an anti-Pgp antibody. An aliquot of each sample before immunoprecipitation was directly probed with the anti-Pgp antibody, to measure the total amount of Pgp in each lysate, and with an anti-pancadherin antibody, used as a control of equal protein loading. The figure shown here is representative of three independent experiments with similar results. C-D. MCF7-dx cells were lysed and the Pgp-rich membrane vesicles were isolated. C. The samples were incubated for 30 min at 37°C in the absence (0) or in the presence of different concentrations of Dox, NitDox, Lip A, Lip B, Caelyx®, to measure the basal ATPase activity. Data are presented as mean ± SD (n = 4). NitDox/Lip A/Lip B/Caelyx® versus Dox: * p < 0.001; Lip A/Lip B versus Caelyx®: ° p < 0.002. D. The samples were incubated for 30 min at 37°C with different concentrations of verapamil (Ver), to measure the basal ATPase activity, in the absence (Ctrl) or in the presence of 5 µM Dox, NitDox, Lip A, Lip B, Caelyx®. Data are presented as mean ± SD (n = 4). For all drugs, versus Ctrl: * p < 0.001; Lip A/Lip B versus Caelyx®: ° p < 0.001.

Besides Pgp, also MRP1, MRP3 and BCRP are involved in Dox efflux1; to assess if free NitDOX and liposomal NitDoxs caused the nitration of other ABC transporters and decreased their catalytic activity, we repeated the assays in HP06 cells, which expressed detectable amounts of all these transporters (Figure 4A). As shown in Supplementary Figure 4A, Pgp, MRP1 and BCRP, but not MRP3, were nitrated by NitDox, Lip A and in particular Lip B. The free NitDox was a weaker nitrating agents than Lip A and Lip B; Dox and Caelyx did not elicit any detectable nitration (Supplementary Figure 4A). Both basal and maximal (verapamil-stimulated) Pgp activity were lower if Pgp was nitrated, i.e. in samples incubated with NitDox, Lip A and in particular Lip B (Supplementary Figure 4B). The same trend was observed for MRP1 (Supplementary Figure 4C); by contrast, we did not measure any reduction of BCRP ATPase activity in each experimental condition (Supplementary Figure 4D).

When we measured the efflux kinetics of Dox from MCF7-dx cells, we did not detect significant changes in the Km for Dox after the treatment with the different formulations (Figure 6A). By contrast, dramatic changes occurred for the Vmax, which progressively decreased...
following this order: Dox > Caelyx® > free Nitdox > Lip A > Lip B (Figure 6A). In keeping with the reduced efflux, Lip B was the only formulation significantly more accumulated than Dox in all the drug-resistant breast and ovary cancer cell lines analyzed; Lip A was more accumulated in all the drug-resistant cells with the exception of the highly resistant HP06 cells (Figure 6B). The free NitDox was more retained in MCF7-dx and A2780-dx cells, but not in MDA-MB-231 and HP06 cells, whereas Caelyx® showed the same accumulation of Dox in drug-resistant cells (Figure 6B). Overall these data suggest that the liposomal NitDoxs were the most effective formulations against breast and ovary Pgp-overexpressing cancer cells, owing to their higher release of NO, which produced strong inhibition of Pgp catalytic activity, decrease of the drug efflux, increased intracellular drug retention and toxicity.

Figure 6. Liposomal NitDoxs have the lowest efflux kinetics and the highest intracellular accumulation

A. MCF7-dx cells were incubated for 20 min with increasing concentrations of Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively), Caelyx®, then the cells were washed and tested for the intracellular drug content. The procedure was repeated on a second series of dishes, incubated in the same experimental conditions and analyzed after 10 min further. Data (mean ± SD, n = 4) are presented as the rate of doxorubicin efflux (dc/dt) plotted versus the initial concentration of the
drug. Vmax (nmol/min/mg proteins) and Km (nmol/mg proteins) were calculated with the Enzfitter software. B. Dox-sensitive (MCF7) and Dox-resistant (MCF7-dx, MDA-MB-231, HP06) breast cancer cells, Dox-sensitive (A2780) and Dox-resistant (A2780-dx) ovary cancer cells were grown for 24 h in fresh medium (Ctrl) or in medium containing 5 µM Dox, nitrooxy-Dox (NitDox), Lip A, Lip B, Caelyx®. The intracellular amount of doxorubicin was measured fluorimetrically. Data are presented as mean ± SD (n = 4). NitDox/Lip A/Lip B/Caelyx® versus Dox: * p < 0.01; Lip A/Lip B versus Caelyx®: ° p < 0.02.

DISCUSSION

In this work we produced and tested two liposomal formulations of NitDox, i.e. NO-releasing Doxs that overcame drug resistance9 and showed different pharmacodynamic properties from parental Dox10. We compared our liposomal formulations with Caelyx®, one of the most used Dox-containing liposomes in the therapy of advanced and metastatic breast and ovary cancers.

Over the last decades, liposomes emerged as effective tools improving the efficacy and decreasing the side effects of chemotherapy treatments. Main aims of these systems are: targeting specific tissues or cells, delivering drugs in a controlled manner, lowering the necessary dose in order to decrease drug toxicity28.

The liposomal NitDoxs used in this work are characterized by different amounts of Chol: Lip A (containing 38.2 % cholesterol) showed a phospholipid composition quite similar to Caelyx®, while in Lip B the Chol amount was halved. These formulations, prepared by thin lipid film hydration and extrusion method, were similar in size and zeta potential values, while the amount of NitDox encapsulated increased with the decrease in Chol amount. The effect of Chol on the entrapment efficiency of hydrophobic molecules can be attributed to molecular interactions between phospholipids, Chol and drug molecules. Both NitDox and Chol prefer to align themselves in the limited space of the hydrophobic region of the membrane bilayer; the competition with Chol for this space explains the lower drug encapsulation when Chol increases29. Several studies have reported lower encapsulation percentages of hydrophobic
molecules (such as paclitaxel and ibuprofen) in the presence of high Chol in the liposome\textsuperscript{30, 31}. The molecular interactions between the drug and the lipid bilayer membrane have great influence on the liposomal formulation process and on the drug release from the liposomes. These interactions determine partitioning, allocation, orientation and conformation of the drug in the bilayer membrane, therein influencing the transport, distribution and accumulation of the drug\textsuperscript{32}.

The DSC studies were performed to further investigate the molecular interaction between the drug and the lipids in relationship to the Chol amount. The incorporation of Chol exerted strong perturbing effects on liposomal NitDoxs: the decrease of liposome $T_m$ in parallel with the increasing Chol concentrations confirmed the marked interaction of NitDox with the bilayer.

Saturated phospholipids producing rigid bilayers are retained in the blood circulation for longer periods when combined with Chol\textsuperscript{33}; we performed \textit{in vitro} stability studies and the results were in accord with the DSC data. The relatively prolonged drug release from liposomes containing higher amount of Chol (Lip A) in comparison to liposomes with lower amount of Chol (Lip B) may be attributed to the bilayer rigidity\textsuperscript{34, 35}. In general, the more rigid is the bilayer, the slower is the release of the drug, in keeping with our analysis of Dox release from Lip A and Lip B, respectively.

Since we first characterized NitDox in human Dox-sensitive colon cancer HT29 cells and in their resistant counterpart HT29-dx cells\textsuperscript{10}, we used these cells as a model for comparing liposomal NitDoxs and free NitDox. The liposomal encapsulation did not change the typical cytosolic localization of free NitDox, a localization markedly different from parental free Dox, which entirely accumulates in the nucleus. Although in the present work we did not investigate the subcellular localization of liposomal NitDoxs, the metabolic effects on mitochondrial metabolism and the mechanisms of cell death, as we did previously for NitDox\textsuperscript{10}, we suppose
that they are shared by free and liposomal NitDoxs. A striking difference between free drug and liposomal formulations was the kinetics of uptake, that increased in a time-dependent manner for both Lip A and Lip B. Of note, the liposomal NitDoxs reached the same accumulation levels in both HT29 and HT29-dx cells, irrespectively of the ABC transporters present in these two cell populations\textsuperscript{10}. By contrast, the NitDox uptake showed a peak at 3 h followed by a plateau in HT29 cells or by a progressive decrease in HT29-dx cells. Although NitDox nitrates the ABC transporters expressed in HT29-dx cells and inhibits their efflux activity\textsuperscript{9, 10}, the decrease in the intracellular accumulation of free NitDox can be explained by the physiological turnover of ABC transporters and by the progressive re-synthesis of new non-nitrated transporters, which may contribute to the drug efflux with renovated efficiency after 24 h. The kinetics of liposomal NitDox accumulation is suggestive of a slower release of NitDox within HT29 and HT29-dx, with a consequent more gradual and prolonged nitration and inhibition of ABC transporters. This hypothesis, which may explain the increased time-dependent accumulation of NitDox released from Lip A and Lip B, was supported by the observation that the liposomal NitDoxs produced a more sustained and prolonged release of nitrite after 48 or 72 h than free NitDox and Dox. Since NO is one of the Dox cytotoxicity mediators\textsuperscript{6, 36}, the higher release of NO can also explain the stronger cytotoxic effects of the liposomal NitDoxs compared with free NitDox or Dox. We cannot discriminate – on the basis of the measurement of nitrite levels – if NO is released from the nitrooxy group of NitDox\textsuperscript{10} or it is produced by the inducible NO synthase (iNOS) activation. Indeed, the higher is the amount of Dox retained within the cells, the higher is the activation of iNOS\textsuperscript{6}, which is capable to produce micromolar concentrations of NO\textsuperscript{37}. Such NO levels exert antitumor effects\textsuperscript{38} and overcome the resistance to Dox in drug-resistant cells, via either ABC transporters-dependent\textsuperscript{6, 39} or independent mechanisms\textsuperscript{40, 41}. Both the direct release of NO from
NitDox (due to the slow release of NitDox from liposomes, followed by the denitrification of NitDox) and the synthesis of NO by iNOS activation (due to the high amount of Dox retained within tumor cells) may contribute to the cytotoxic activity exerted by the liposomal NitDoxs in drug-resistant cells.

Whatever the source of NO, it is interesting to note that the effects of Lip B in HT29-dx cells, in terms of cytotoxicity and NO release, were stronger than those of Lip A. This difference can be explained by the slightly faster release of NitDox from Lip B, owing to the lower rigidity of the liposomal bilayer.

Since liposomal NitDoxs were more cytotoxic than free NitDox against tumor cells, we wondered whether they also exerted increased cytotoxicity in cardiomyocytes, the main target of Dox-associated adverse effects. In our model, the liposomal NitDoxs released more NO than free Dox and NitDox, but they did not produce a higher cytotoxicity. The role of NO in Dox-induced cardiotoxicity or cardioprotection is highly controversial. Interestingly, a dietary supplementation with nitrates, the same form of NO derivatives released from NitDox, exerted a cardioprotective role in patients treated with Dox. Although on the basis of our in vitro tests we cannot infer any direct information about the cardiotoxicity of liposomal NitDoxs in vivo, it has been widely proved that the liposomal Dox formulations are less cytotoxic than the free drug. In dose-dependence experiments, liposomal NitDoxs exerted stronger antitumor toxicity than free NitDox and Dox at the highest concentration (5 µM). We might speculate that liposomal NitDoxs share with the clinically used liposomal Dox formulations a lower cardiotoxicity coupled with a higher antitumor efficacy. Intriguingly, the liposomal NitDoxs accumulated at a lower extent than free Dox in cardiomyocytes, whereas the opposite trend was observed in colon cancer cells. Free Dox enters by passive diffusion or by carrier-mediated transport; by contrast,
the uptake of liposomal drugs is essentially mediated by endocytosis. Different cell types vary for the basal endocytic rate, the lipid composition and the physico-chemical properties of plasma-membrane: such differences may determine a different uptake of liposomal drugs versus free drugs. Once uptaken by the cells, Dox may: reach its targets, be metabolized and inactivated, be sequestered in endo-lysosomes and then subjected to exocytosis, be effluxed by ABC transporters. Free Dox is immediately available for these processes once entered within the cytosol; liposomal Dox is gradually released from the liposomal envelope and its pharmacodynamic/kinetic effects are delayed compared to free Dox\textsuperscript{14, 15}. All these differences affect amount and timing of the intracellular retention of liposomal and free drugs in the tissues, and may explain the variable behavior of liposomal NitDoxs versus free Dox in H9c2, HT29 and HT29-dx cells.

Despite the increasing number of evidences reporting that liposomal formulations overcome chemoresistance in tumors\textsuperscript{11-13}, it has been shown that Caelyx\textsuperscript{®} and liposomal-based formulations of Dox had the same efficacy of Dox in drug-resistant tumors\textsuperscript{45}. Keeping in mind this observation, we compared the efficacy of our liposomal NitDox formulations with the efficacy of Caelyx\textsuperscript{®} in a panel of human breast and ovary cancer cells, with a different degree of Dox-resistance and a different pattern and expression of ABC transporters. Such panel includes cells with different types of drug resistance (i.e. drug-induced and constitutive resistance) and with different origin (i.e. immortalized cell lines and primary tumor cells).

Liposomal NitDox formulations were not more cytotoxic than Dox, free NitDox or Caelyx\textsuperscript{®} in drug-sensitive cells (MCF7, A2780), but they were more effective than Caelyx\textsuperscript{®} in MCF7-dx resistant cells. We did not detect appreciable differences between Caelyx\textsuperscript{®} and Lip A, which contained the same Chol concentration of Caelyx\textsuperscript{®}, in the other Dox-resistant cells; only Lip B
induced significant toxicity in all the cell lines analyzed, including the primary breast cancer HP06 cells, which had the strongest drug-resistant phenotype.

Our data about cytotoxicity are in agreement with the observation that Doxyl® is not effective in drug-resistant MCF7/Adr and A2780/Dox cells, and exerts only a moderate increase of cytotoxicity when associated with Pluronic block copolymers P85. Since Pluronic likely interferes with the efficiency of efflux transporters, we focused on this issue. Interestingly, liposomal NitDoxs produced a clearly detectable nitration of Pgp isolated from MCF7-dx cells. This nitration was higher than the one induced by free NitDox. No nitration was evident on Pgp extracted from MCF7-dx cells treated with Dox, a hardly detectable nitration was observed after the exposure of these cells to Caelyx®. This trend was in accord with the different amount of NO produced by each drug in the Dox-resistant cell populations: only liposomal NitDoxs and – to a lesser extent and only in some cell lines – free NitDox increased the nitrite levels. By contrast, Caelyx® was as ineffective as Dox in increasing NO. In consequence of the nitration, the catalytic ATPase activity of Pgp was reduced: again, only drugs releasing huge amounts of NO – i.e. liposomal NitDoxs and to a lesser extent free NitDox – strongly inhibited Pgp catalytic activity, contrarily to Caelyx® and Dox. Pgp is not the only target of liposomal NitDoxs: MRP1 and BCRP were nitrated in HP06 cells, which expressed also these transporters. Interestingly, whereas the activity of Pgp and MRP1 was reduced following the nitration, the activity of BCRP was unmodified, excluding that the nitration of this protein affects Dox efflux. We hypothesize that, in cells expressing Pgp and MRP1, the nitration of both transporters is responsible for the increased retention of Dox.

According to the kinetics of efflux from whole MCF7-dx cells, the Vmax of Dox released from liposomal NitDoxs was markedly lower than that of Caelyx®: therein, Dox derived from
liposomal NitDoxs was more retained within the cells, as confirmed by the fluorimetric quantification of Dox in the panel of resistant cells. The free NitDox showed an efflux rate and an intracellular accumulation falling between those observed for liposomal NitDoxs and Caelyx®, in keeping with its intermediate ability of increasing NO levels. Since the greatest differences among each Dox formulation concerned the Vmax and not the Km, we hypothesize that NO acts as a non competitive inhibitor of Pgp, i.e. it would limit the maximal efficiency of the enzyme without changing its affinity for Dox.

Again, primary HP06 cells were the most refractory to exhibit increased levels of NO and intracellular Dox accumulation: only Lip B produced a small but significant increase of nitrite and intracellular Dox. This increase may be due to the faster drug release from this formulation, that allows higher delivery of NitDox within resistant cells, higher release of NO, higher inhibition of Pgp.

These results confirm the advantages of using liposomes as drug carriers, owing to their sustained release of Dox. In particular Lip B, the liposomal NitDox containing a lower amount of Chol, produced the most interesting results: this formulation is characterized by a good NitDox entrapment efficiency and by an interesting drug release profile that determines a strong cytotoxic potential against drug-resistant cells.

In summary, we propose the liposomal NitDoxs formulation as new tools with a specific indication for Pgp- and MRP1-overexpressing tumors. Liposomal NitDoxs may provide at least two benefits: 1) being liposomal formulations, they should exert a higher drug accumulation in tumors, due to the EPR effect and the lower cardiotoxicity, as it occurs for Caelyx® or Doxyl®; 2) being stronger releasers of NO, they can inhibit the efflux activity of ABC transporters and accumulate at cytotoxic levels in Dox-resistant tumors. The use of NO donors as antitumor
adjuvant therapy is under investigation\textsuperscript{47} and the first phase II clinical trials have been completed\textsuperscript{48}. The present work adds a step in this direction. By coupling the innovation of a multi-target chemotherapeutic agent with the advantage of liposomal formulations, our results suggest that this strategy can be particularly promising against drug-resistant tumors and pave the way to the further development of liposomal NitDoxs \textit{in vivo}, against tumors unresponsive to Dox and to the clinically available Dox liposomal formulations.

ACKNOWLEDGMENT

We are grateful to Prof. Roberta Cavalli (Department of Drug Science and Technology, University of Torino) for DSC analysis. We are grateful to Mr. Costanzo Costamagna (Department of Oncology, University of Torino) and Dr. Erika Ortolan (Department of Medical Science, University of Torino) for the technical assistance. We thank Prof. Alberto Gasco for the fruitful discussions.

This work has been supported by grants from the Italian Ministry of University and Research (program Future in Research FIRB 2012), Italian Association for Cancer Research (AIRC; MFAG 11475).

Joanna Kopecka is the recipient of a FIRC (Fondazione Italiana per la Ricerca sul Cancro) fellowship.

SUPPORTING INFORMATION AVAILABLE

Supporting Information include Supplementary Figures 1, 2, 3 and 4. This information is available free of charge via the Internet at http://pubs.acs.org/.
REFERENCES:


Supplementary Figure 1. Free NitDox and liposomal NitDox have a cytosolic localization

HT29 cells were incubated with 5 μM Dox, nitrooxy-Dox (NitDox), or a liposomal formulation of Nitdox containing 19.1% Chol (Lip B). After 24 h, cells were washed, fixed, counterstained with the nuclear dye DAPI and analyzed for the intracellular localization of Dox by fluorescence microscopy. The liposomal formulation of Nitdox containing 38.2% Chol (Lip A) had the same localization of Lip B (not shown in the figure). Magnification: 63 x objective; 10 x ocular lens. The micrographs are representative of 3 experiments with similar results. Bar: 10 μm.
Supplementary Figure 2. Cytotoxicity and nitrite levels in drug-sensitive and drug-resistant cells treated with different concentrations of Dox, free NitDox and liposomal NitDox

A-B. HT29 cells and doxorubicin-resistant HT29-dx cells were incubated with different concentrations of Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively) for 24 h. Cell supernatants were collected and checked spectrophotometrically for the release of LDH (panel A), taken as an index of cytotoxicity, and for the amount of nitrite (panel B), taken as an index of NO release. Data are presented as mean ± SD (n = 3). For all panels and all drugs, versus untreated (0) cells: * p < 0.05; Lip A/Lip B/NitDox versus Dox: ˙ p < 0.02; Lip A/Lip B versus NitDox: ° p < 0.05.
Supplementary Figure 3. Liposomal NitDoxs are less cytotoxic than Dox in H9c2 cardiomyocytes

H9c2 cells were grown in fresh medium (Ctrl) or incubated in medium containing 5 μM Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively) for 24, 48, 72 h. A-B. At each time point, cell supernatants were collected and checked spectrophotometrically for the release of LDH (panel A), and for the amount of nitrite (panel B). Data are presented as mean ± SD (n = 3). Versus untreated (Ctrl) cells: * p < 0.001; Lip
A/Lip B/NitDox versus Dox: ◇ p < 0.005. C. At each time point, cells were lysed and the intracellular amount of Dox was quantified fluorimetrically. Data are presented as mean ± SD (n = 3). For all drugs, versus untreated (0) cells: * p < 0.001; Lip A/Lip B versus Dox: ◇ p < 0.001; Lip A/Lip B versus NitDox: ◇ p < 0.001.
## Supplementary Figure 4

### A

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>Dox</th>
<th>Nitdox</th>
<th>Lip A</th>
<th>Lip B</th>
<th>Caelyx</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitro-Pgp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pgp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitro-MRP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitro-MRP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRP3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitro-BCRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pncadherin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

**Pgp ATPase activity (μmol/min/mg prot)**

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>NitDox</th>
<th>Lip A</th>
<th>Lip B</th>
<th>Pgp ATPase activity (μmol/min/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

**MRP1 ATPase activity (μmol/min/mg prot)**

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>NitDox</th>
<th>Lip A</th>
<th>Lip B</th>
<th>MRP1 ATPase activity (μmol/min/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

**BCRP ATPase activity (μmol/min/mg prot)**

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>NitDox</th>
<th>Lip A</th>
<th>Lip B</th>
<th>BCRP ATPase activity (μmol/min/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
Supplementary Figure 4. Effects of liposomal NitDoxs on nitrization and activity of ABC transporters

Dox-resistant breast cancer cells HP06 were grown for 24 h in fresh medium (Ctrl) or in medium containing 5 µM Dox, nitrooxy-Dox (NitDox), liposomal formulations of NitDox containing 38.2% or 19.1% Chol (Lip A or Lip B, respectively), Caelyx®. A. Cells were lysed and immunoprecipitated with an anti-nitrotyrosine antibody, then probed with anti-Pgp, anti-MRP1, anti-MRP3, anti-BCRP antibodies. An aliquot of each sample before immunoprecipitation was directly probed with the above mentioned antibodies, to measure the total amount of Pgp, MRP1, MRP3, BCRP in each lysate, and with an anti-pancadherin antibody, used as a control of equal protein loading. The figure shown here is representative of three independent experiments with similar results. B. Cells were lysed and the Pgp-rich membrane vesicles were isolated. Left panel: samples were incubated for 30 min at 37°C in the absence (Ctrl) or in the presence of 5 µM NitDox, Lip A, Lip B, to measure the basal ATPase activity. Right panel: samples were incubated for 30 min at 37°C in the absence (Ctrl) or in the presence of 5 µM NitDox, Lip A, Lip B, plus 20 µM verapamil, to measure the maximal ATPase activity. Data are presented as mean ± SD (n = 4). Versus Ctrl: * p < 0.005. C-D. 50 µg of immunoprecipitated MRP1 (panel C) or BCRP (panel D) were incubated for 30 min at 37°C in the absence (Ctrl) or presence of 5 µM NitDox, Lip A, Lip B, then the ATPase activity of each transporter was measured. Data are presented as mean ± SD (n = 4). Versus Ctrl: * p < 0.01.