

# Pleiotropic activities of nitric oxide-releasing doxorubicin on P-glycoprotein/ABCB1

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Doxorubicin is one of the first-line chemotherapeutic drugs for osteosarcoma, but the rate of success is below 60% of patients. The main cause of this low success is the presence of P-glycoprotein (P-gp/ABCB1) that effluxes the drug, limiting the intracellular accumulation and toxicity of Doxorubicin. P-gp also inhibits immunogenic cell death promoted by Doxorubicin. Nitric oxide-releasing Doxorubicin is a synthetic anthracycline effective against P-gp-positive osteosarcoma cells. It is not known how it impacts on P-gp expression and immunogenic cell death induction. To address this point, we treated human Doxorubicin-sensitive osteosarcoma U-2OS cells and their resistant variants with increasing amount of P-gp, with Dox and Nitric oxide-releasing Doxorubicin. While Doxorubicin was cytotoxic only in U-2OS cells, Nitric oxide-releasing Doxorubicin maintained its cytotoxic properties in all the resistant variants. Nitric oxide-releasing Doxorubicin elicited a strong nitrosative stress in whole cell extracts, endoplasmic reticulum and plasma membrane. P-gp was nitrated in all these compartments. The nitration caused protein ubiquitination and lower catalytic efficacy. The removal of P-gp from cell surface upon Nitric oxide-releasing Doxorubicin treatment disrupted its interaction with calreticulin, an immunogenic cell death-inducer that is inhibited by P-gp. Drug resistant cells treated with Nitric oxide-releasing Doxorubicin exposed calreticulin, were phagocytized by dendritic cells and expanded anti-tumor CD8<sup>+</sup> T-lymphocytes. The efficacy of Nitric oxide-releasing Doxorubicin was validated in Dox-resistant osteosarcoma xenografts and was higher in immune-competent humanized mice than in immune-deficient mice, confirming that part of Nitric oxide-releasing Doxorubicin efficacy relies on the restoration of immunogenic cell death. Nitric oxide-releasing Doxorubicin was a pleiotropic anthracycline reducing activity and expression of P-gp, and restoring immunogenic cell death. It can be an innovative drug against P-gp-expressing/ Doxorubicin-resistant osteosarcomas.

## Keywords

Osteosarcoma; doxorubicin; nitric oxide; P-glycoprotein; calreticulin; immunogenic cell death

## 1. Introduction

Osteosarcoma is a common tumor in childhood and adolescent age. The golden standard of treatment is surgery coupled with neo-adjuvant and adjuvant chemotherapy, based on doxorubicin (Dox), cisplatin and methotrexate [1]. The average efficacy of chemotherapy is around 60% [2], because osteosarcoma is characterized by multiple mechanisms of drug resistance. The overexpression of the detoxifying enzyme glutathione-S-transferase p1 determines resistance to cisplatin [3]. The reduced expression of the folate carrier SLC19A1 [4, 5, 6] or the folate metabolizing enzyme folylpoly- $\gamma$ -glutamate synthetase [7] causes resistance to methotrexate. P-glycoprotein (P-gp) is the main determinant of resistance to Dox, which is actively effluxed by this plasma membrane-associated transporter [8]. Indeed, P-gp is recognized as a robust negative clinical prognostic factor in osteosarcoma [9]. In the last years, three generations of P-gp inhibitors have been produced [10]. Although promising *in vitro*, most inhibitors failed in patients because of the poor specificity, the undesired toxicity, the unfavorable interactions with other drugs and pharmacokinetic profile [11].

Boosting the anti-tumor activity of dendritic cells (DCs) [12] or cytotoxic CD8<sup>+</sup> T-lymphocytes [13], using CAR T-cells [14] or immune-checkpoint inhibitors [15] have been tested in pre-clinical models or clinical trials as alternative strategies in the treatment of osteosarcoma. Currently, these immune-therapy-based approaches do not offer a significant advantage compared to chemotherapy [16].

Specific chemo-immune-therapy protocols may improve the efficacy of antitumor treatments. For instance, trabectedin, a drug employed in sarcomas and ovarian cancers, reduces osteosarcoma growth and metastases by recruiting CD8<sup>+</sup> T-lymphocytes. These CD8<sup>+</sup> T-lymphocytes, however, express high levels of the immune-suppressive checkpoint PD-1 [17], raising doubts on their effective functionality. Dox is a chemotherapeutic drug inducing immunogenic cell death (ICD): it increases the translocation - from endoplasmic reticulum (ER) to plasma membrane - of calreticulin (CRT), a molecule that stimulates the phagocytosis of tumor cells by DCs and expands anti-tumor CD8<sup>+</sup> T-lymphocytes [18]. However, the presence of P-gp impairs Dox-mediated ICD for at least two reasons. First, P-gp-expressing cells have low accumulation of Dox that is insufficient to trigger ICD. Second, P-gp co-localizes with CRT on plasma membrane, impairing its

immune-sensitizing functions [19, 20]. Dox fails to induce ICD in resistant/P-gp-expressing osteosarcoma cells [21], and this failure mediates part of the resistance to the drug. In a previous work, we demonstrated that synthetic Dox with tropism for ER and ability to perturb protein folding by releasing nitric oxide (NO) or H<sub>2</sub>S are cytotoxic against P-gp-expressing osteosarcoma cells. These synthetic Doxs also induce ICD [22].

NO is an inhibitor of the catalytic activity of P-gp: by reacting with superoxide (O<sub>2</sub><sup>•-</sup>) NO generates peroxynitrite (ONOO<sup>-</sup>) that nitrates both nucleic acids and proteins on tyrosine [23]. One of the nitrated protein is P-gp, which reduces its catalytic activity. This event increases the retention of Dox in P-gp-expressing myeloid leukemia cells, malignant pleural mesotheliomas and ovary cancers and facilitates Dox-induced ICD [24]. It has not been fully elucidated if nitric oxide-releasing Dox (NODox) restores Dox cytotoxicity and ICD by affecting P-gp amount, e.g. by inducing protein unfolding or destabilization, besides affecting P-gp activity.

The aim of this study is to analyze if NODox may affect stability and expression of P-gp in human Dox-resistant osteosarcoma cells, and improve tumor killing by restoring a complete ICD.

## 2. Materials and Methods

### 2.1 Chemicals

Fetal bovine serum and culture medium were purchased by Invitrogen Life Technologies (Carlsbad, CA), plastic ware by Falcon (Becton Dickinson, Franklin Lakes, NJ), reagents for electrophoresis by Bio-Rad Laboratories (Hercules, CA). BCA kit (Sigma-Merck-Millipore, St. Louis, MO) was used to measure the protein concentration. Dox was purchased from Sigma-Merck-Millipore. NODox was synthesized as reported in [25]. All other reagents were from Sigma-Merck-Millipore.

### 2.2 Cell lines

Human Dox-sensitive osteosarcoma U-2OS cells were from ATCC (Manassas, VA). From parental cells, variants with increasing resistance to Dox (U-2OS/DX30, U-2OS/DX100, U-2OS/DX580), were generated after culturing cells in medium with increasing concentrations of Dox and then maintaining them in medium with 30, 100, 580 ng/ml Dox [26]. U-2OS/DX580 CRT<sup>+</sup> cells were generated by transfecting U-2OS/DX580 cells with 1 µg of CRT expressing vector (NM\_004343; Origene, Rockville, MD), as per manufacturer's instructions. Cells were then selected in 1 µg/ml neomycin-containing medium for 3 weeks. Cell authentication was performed using the microsatellite analysis method, with the PowerPlex kit (Promega Corporation, Madison, WI; last authentication: January 2020).

### 2.3 Dox accumulation

Dox accumulation was measured by a fluorimetric assay [27], using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Winoosky, MT). The results were expressed as nmoles Dox/mg proteins, according to the calibration curve.

### 2.4 Apoptosis and cell viability

Apoptosis was measured fluorimetrically, by measuring the activation of caspase 3, indicated by the cleavage of the fluorogenic substrate Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) [20]. Fluorescence was read using a Synergy HT

Multi-Detection microplate reader (Bio-Tek Instruments) and converted in nmoles AMC/mg cellular proteins, using a calibration curve of AMC solutions. Cell viability was evaluated by the AT-Plite Luminescence Assay System (PerkinElmer, Waltham, MA). Results were expressed as percentage of viable cells relative to untreated cells, considered 100% viable.

### 2.5 Nitrite measurement

The amount of nitrite, the stable derivative of NO, was measured spectrophotometrically in the medium by the Griess method [27], using a Packard EL340 microplate reader (Bio-Tek Instruments). Results were expressed as nmoles nitrite/mg cellular proteins, using a calibration curve.

### 2.6 Nitrotyrosine measurement

The amount of nitrotyrosines, an index of nitrosative stress, was measured in whole cell lysates, ER extracts or plasma membrane extracts, using the Nitrotyrosine ELISA kit (Hycult Biotechnology, Uden, The Netherlands). ER and plasma membrane extracts were isolated with the Endoplasmic Reticulum Isolation Kit (Sigma-Merck-Millipore) and the Cell Surface Protein Isolation kit (Thermo Fisher Scientific Inc., Rockford, IL), respectively. The absorbance was read with a Packard EL340 microplate reader and converted into pmoles nitrotyrosine/mg proteins according to the titration curve.

### 2.7 Immunoblotting

Cells were rinsed with MLB buffer (Millipore, Burlington, MA), sonicated and centrifuged at 13,000 × g for 10 min at 4 °C. Fifty µg of proteins were resolved by 4 - 15% SDS-PAGE and probed with an anti-ABCB1/P-gp (clone C219, Millipore, diluted 1 : 500) or an anti-β-tubulin (clone D10, Santa Cruz Biotechnology Inc., Santa Cruz, CA, diluted 1 : 1,000) antibody, followed by incubation with the secondary peroxidase-conjugated antibodies (Bio-Rad) and detection by enhanced chemiluminescence (Bio-Rad). ER-associated proteins were extracted with the Endoplasmic Reticulum Isolation Kit; 50 µg proteins were probed for P-gp or CRT (PA3-900, ABR-Affinity BioReagents Inc., Golden, CO, diluted 1 : 500). Biotinylation assays were used to measure the plasma membrane-associated proteins, using the Cell Surface Protein Isolation kit [24]; 50 µg proteins were probed with anti-P-gp, anti-CRT or anti-pancadherin antibody (clone CH-19, diluted 1 : 500, Santa Cruz Biotechnology Inc.). For P-gp ubiquitination, 50 µg proteins were immuno-precipitated with the PureProteome protein A and protein G Magnetic Beads (Millipore) in the presence of the anti-P-gp antibody, then immunoblotted with an anti-mono/polyubiquitin antibody (clone FK2, diluted 1 : 1,000, Axxora, Lausanne, Switzerland,) [20]. In co-immunoprecipitation assays, 100 µg of plasma membrane-associated proteins were immuno-precipitated with the anti-CRT antibody, then blotted for P-gp [20].

### 2.8 P-gp ATPase activity

The rate of ATP hydrolysis, an index of the catalytic cycle of Pgp, was measured after immunoprecipitating Pgp from 100 µg of membrane-associated proteins, in a spectrophotometric assay [28]. Results were expressed as nmoles hydrolyzed phosphate (Pi)/mg proteins.

## 2.9 Phagocytosis and T-lymphocyte activation

Monocytes, isolated using the Pan Monocyte isolation Kit (Miltenyi Biotec., Tetrow, Germany) from the peripheral blood of healthy donors (Blood Bank of AOU Città della Salute e della Scienza, Torino, Italy), were used to generate DCs, as previously reported [29]. The phagocytosis assay was carried out as detailed in [18], using DC-tumor cells co-cultures at 37 °C and 4 °C for 24 h. Cells phagocytized at 4 °C were subtracted from the cells phagocytized at 37 °C and were always below 5% (not shown). Phagocytic index was used to express the rate of phagocytosis [18]. After phagocytosis, DCs-autologous T-cells co-cultures were set up for 10 days, after isolating T-lymphocytes with the Pan T Cell Isolation Kit (Miltenyi Biotec.). The percentage of CD8<sup>+</sup> CD107<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells, a signature identifying activated cytotoxic T-lymphocytes [29, 30], was measured by flow cytometry, employing anti-human fluorescein isothiocyanate (FITC)-conjugated-CD8 (clone BW135/80, diluted 1 : 10), phycoerythrin (PE)-conjugated-CD107 (clones H4A3, diluted 1 : 10), allophycocyanine (APC)-conjugated INF- $\gamma$  (REA600, diluted 1 : 10) antibodies (Miltenyi Biotec.). Experiments were carried out with Guava® easyCyte flow cytometer (Millipore) and InCyte software (Millipore).

## 2.10 In vivo tumor growth

100  $\mu$ l of  $1 \times 10^7$  U-2OS/DX580 cells, re-suspended in Matrigel, were injected subcutaneously (s.c.) in 6-week old female NOD SCID gamma mice (NSG) or in NSG mice engrafted with human hematopoietic CD34<sup>+</sup> cells (Hu-CD34<sup>+</sup>; The Jackson Laboratories, Bar Harbor, MA). Mice (5/cage) were housed following 12 h light/dark cycles, with drinking and food *ad libitum*. Tumor growth was monitored daily by caliper and calculated as:  $(L \times W^2)/2$ , where L = tumor length and W = tumor width. When tumors had a 50 mm<sup>3</sup> volume, mice were randomized and treated as it follows (on day 3, 9 and 15 after randomization): 1) vehicle group, treated with 0.1 ml saline solution intravenously (i.v.); 2) Dox group, treated with 5 mg/kg Dox i.v.; 3) NODox group, treated with 5 mg/kg NODox i.v. Tumor volumes and mice weight were monitored daily. Zolazepam (0.2 ml/kg) and xylazine (16 mg/kg) were used for euthanasia on day 21. Tumors were excised and weighted. The Bio-Ethical Committee of the Italian Ministry of Health (#122/2015-PR) approved the experimental protocol.

## 2.11 Statistical analysis

Results are expressed as means  $\pm$  SD and evaluated with a one-way analysis of variance (ANOVA), with the Statistical Package for Social Science (SPSS) software (IBM SPSS Statistics v. 19).  $P < 0.05$  was considered statistically significant.

# 3. Results

## 3.1 Nitric oxide-releasing doxorubicin is cytotoxic in P-gp-expressing human osteosarcoma cells

Dox and NODox (Fig. 1A) were evaluated for their cytotoxic potential in human Dox-sensitive U-2OS cells and in the U-2OS/DX30, U-2OS/DX100 and U-2OS/DX580 sublines, with increasing amount of P-gp (Fig. 1B). As shown in Fig. 1C, the intracellular Dox accumulation was progressively lower in the resistant sublines, while the amount of NODox was the same, independently on the levels of P-gp. In all the resistant variants, intracellular NODox remained significantly higher than Dox. Consistently,

Dox triggered apoptosis, i.e. it activated caspase 3 (Fig. 1D), and decreased cell viability (Fig. 1E) in U-2OS cells only. By contrast, NODox induced apoptosis and decreased the number of viable cells without differences between sensitive and resistant cells (Fig. 1D-E). When accumulated within the cell, Dox is able to increase the synthesis of NO, by up-regulating NO synthase 2 (NOS2) gene: this mechanism mediates part of the Dox anti-cancer effects [24]. In line with the intracellular accumulation, Dox increased nitrite, the NO stable derivative [27], only in U-2OS cells. This property was progressively lost in the resistant variants (Fig. 1F). On the contrary, nitrite amount after treatment with NODox was always significantly higher than the amount detected in the supernatants of untreated cells. Moreover, nitrite did not differ between Dox-sensitive and Dox-resistant cells after treatment with NODox (Fig. 1F), which releases NO directly from its NO donor moiety [25].

To investigate if and how the increased levels of NO impact on P-gp expression, we focused on Dox-sensitive U-2OS cells and on U-2OS/DX580 cells, i.e. the most Dox-resistant variant.

## 3.2 Nitric oxide-releasing doxorubicin induces P-gp nitration followed by ubiquitination

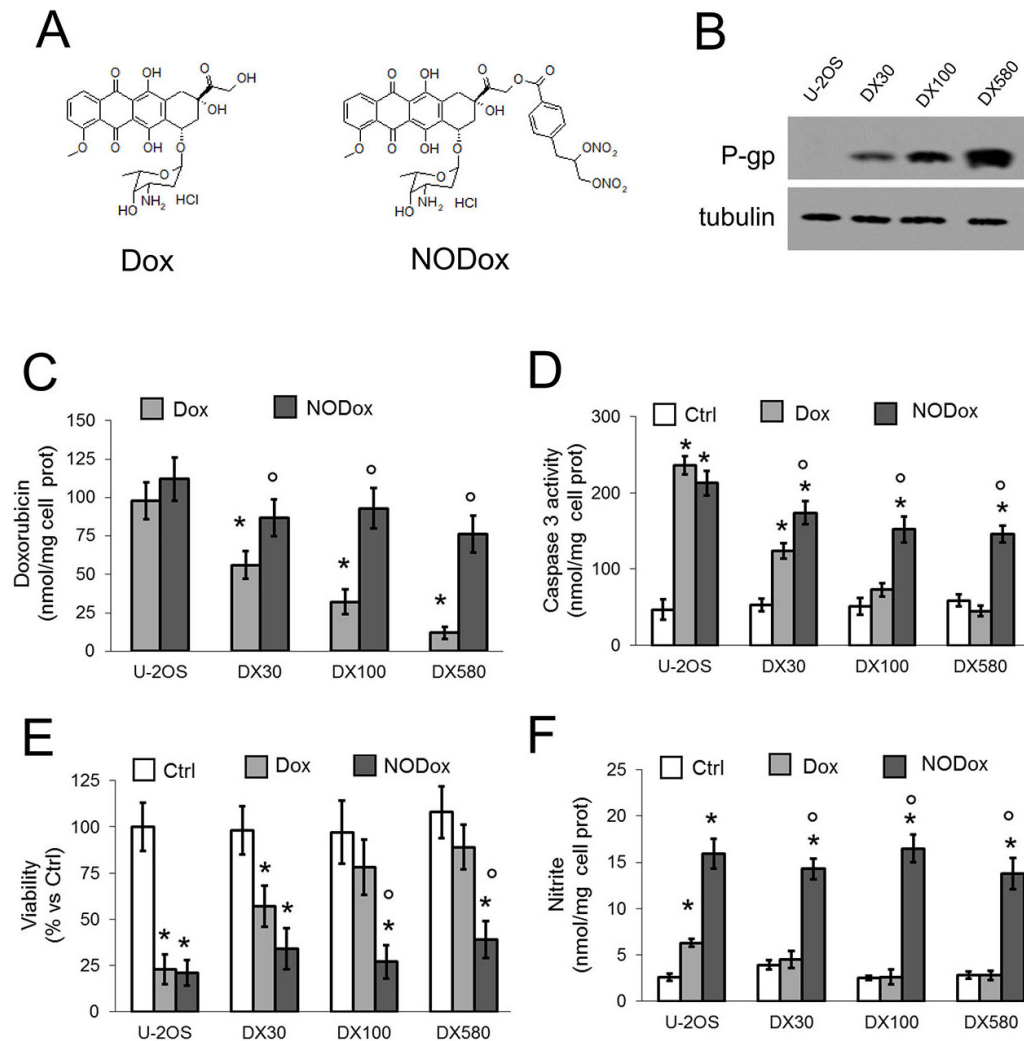
The release of NO and the generation of ONOO<sup>-</sup> induce a nitrosative stress that may alter folding, stability and activity of the target proteins [31]. While Dox increased the amount of nitro-tyrosines in whole cell lysate from U-2OS cells but not from U-2OS/DX580 cells, NODox induced a robust and comparable nitration in both cell lines (Fig. 2A). In U-2OS cells, where the levels of P-gp were undetectable, we did not find any nitration of the protein, neither by Dox nor by NODox. By contrast, NODox induced the nitration of P-gp in U-2OS/DX580 variant, where the protein was abundantly expressed (Fig. 2B).

P-gp is synthesized and folded within ER, then it undergoes glycosylation in the Golgi apparatus and migrates to the plasma membrane [19, 32]. Notably, the same pattern of nitrotyrosines observed in whole cell lysates was detected in ER (Fig. 2C) and plasma membrane (Fig. 2D) extracts: Dox elicited a small nitration only in U-2OS cells, NODox induced a stronger nitration in U-2OS cells and U-2OS/DX580 cells. Accordingly, we did not detect any nitration of P-gp in U-2OS cells, nor in U-2OS/DX580 cells treated with Dox in ER and plasma membrane (Fig. 2E-F). Instead, a clear nitration of P-gp was present in both compartments of U-2OS/DX580 cells treated with NODox (Fig. 2E-F).

Notably, while Dox did not alter P-gp levels in U-2OS/DX580 cells, NODox reduced it in whole cell extracts (Fig. 2B), as well as in ER (Fig. 2E) and plasma membrane (Fig. 2F) fractions. PTIO, a NO scavenger that abrogated the increase of nitro-tyrosines induced by NODox (Fig. 2G), also prevented the nitration and the decrease in the amount of P-gp in U-2OS/DX580 cells (Fig. 2H).

The down-regulation of P-gp was paralleled by the increase in its ubiquitination: while in U-2OS cells we did not detect any sign of ubiquitination, a band of approximately 170 kDa, corresponding to the mono-ubiquitinated form of P-gp was visible in U-2OS/DX580 cells. NODox treatment induced also a strong poly-ubiquitination (Fig. 3A). In parallel, NODox, but not Dox, produced a significant decrease in the catalytic activity of P-gp from U-2OS/DX580 extracts (Fig. 3B).

Overall, these data suggest that NODox decreases P-gp activity



**Fig. 1. NO-releasing doxorubicin is cytotoxic against P-gp-expressing human osteosarcoma cells.** A. Chemical structures of Dox and NODox. B. Expression of P-gp in human Dox-sensitive U-2OS cells and Dox-resistant U-2OS/DX30, U-2OS/DX100 and U-2OS/DX580 variants. The  $\beta$ -tubulin expression was used as control for equal protein loading. The figure is representative of 3 independent experiments. In the following panels, cells were incubated for 24 h (panels C, D, F) or 48 h (panel E) in fresh medium (Ctrl) or in medium containing 5  $\mu$ M Dox or 5  $\mu$ M NODox. C. The intracellular Dox accumulation was measured fluorimetrically, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : DX-cells vs. U-2OS cells; ° $P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells. D. The activation of caspase 3 was measured fluorimetrically, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox/NODox-treated cells vs. Ctrl cells; ° $P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells. E. Percentage of viable cells, measured by a chemiluminescence-based assay, in quadruplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox/NODox-treated cells vs. Ctrl cells; ° $P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells. F. Nitrites were measured spectrophotometrically, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox/NODox-treated cells vs. Ctrl cells; ° $P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells.

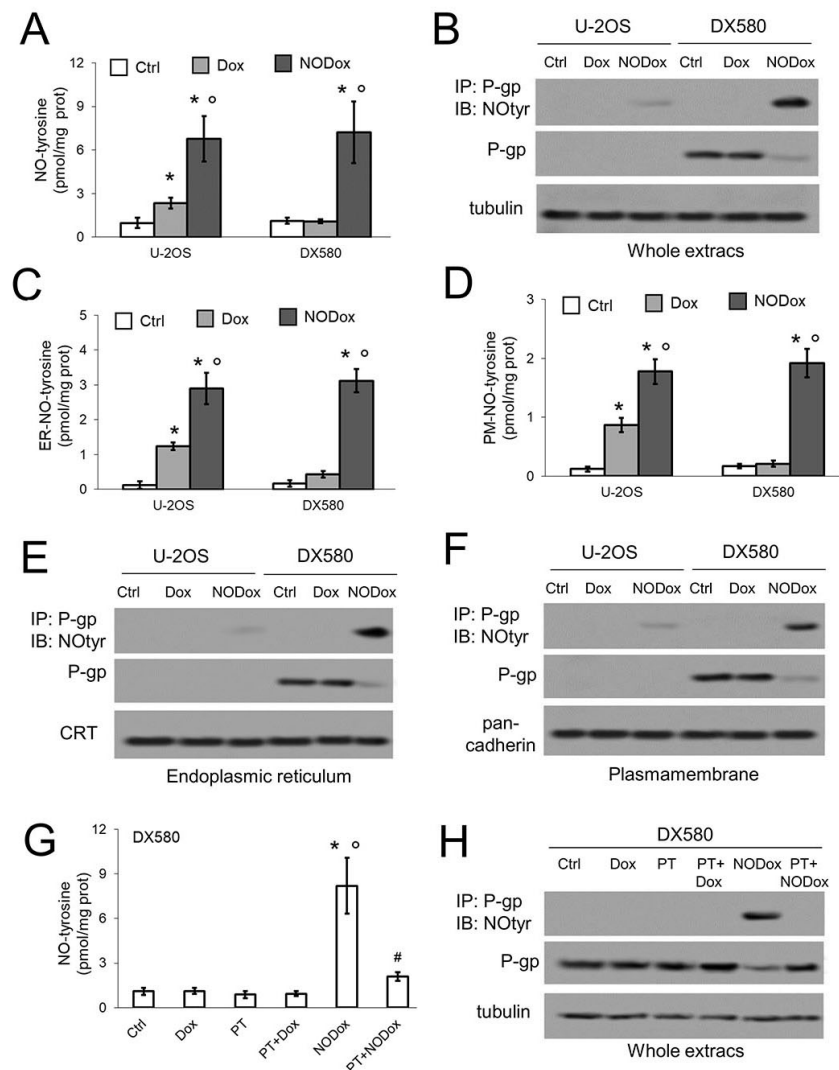
and amount by inducing nitration and ubiquitination of the protein. NO triggers both processes, as demonstrated by the reduced ubiquitination (Fig. 3A) and restored ATPase activity (Fig. 3B) in resistant cells treated with PTIO.

### 3.3 By lowering the amount of P-gp, nitric oxide-releasing doxorubicin restores the immunogenic cell death in drug resistant osteosarcomas

Since P-gp prevents the ICD induced by CRT [24], we next investigated whether the reduction of P-gp elicited by NODox resulted in the restoration of ICD. In U-2OS cells, both Dox and NODox elicited the translocation of CRT on plasma membrane; in U-

2OS/DX580, only NODox-- that was well retained within the cells, differently from Dox-- elicited the exposure of CRT (Fig. 3C). When constitutively overexpressed in U-2OS/DX580 cells, CRT co-immunoprecipitated with P-gp, as already reported in breast cancer P-gp-positive cells [24]. In U-2OS cells we did not detect any interaction between P-gp and CRT, because these cells had undetectable amount of P-gp (Fig. 1B). In P-gp-rich U-2OS/DX580 cells, neither after the treatment with Dox nor after the treatment with NODox we observed a co-immunoprecipitation between P-gp and CRT (Fig. 3C): in Dox-treated cells, CRT was not translocated; in NODox-treated cells, P-gp was dramatically reduced on





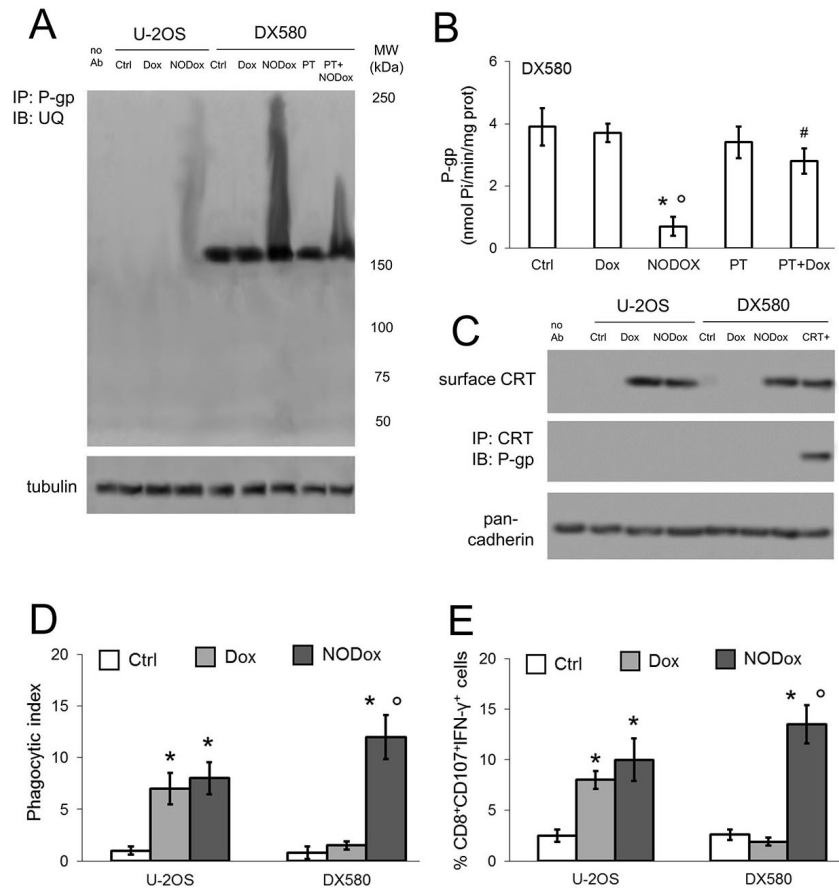
**Fig. 2. NO-releasing doxorubicin induces tyrosine nitration of P-gp in different cellular compartments.** Human Dox-sensitive U-2OS cells and Dox-resistant U-2OS/DX580 cells were incubated for 24 h in fresh medium (Ctrl) or in medium containing 5  $\mu$ M Dox or 5  $\mu$ M NODox. When indicated, the NO scavenger PTIO (100  $\mu$ M, PT) was co-incubated. A. ELISA detection of nitrotyrosines in whole cell extracts, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox/NODox-treated cells vs. Ctrl cells;  $^{\circ}P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells. B. Expression of P-gp in whole cell extracts. The  $\beta$ -tubulin expression was used as control for equal protein loading. The figure is representative of 3 independent experiments. C. ELISA detection of nitrotyrosines in ER-derived extracts, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox/NODox-treated cells vs. Ctrl cells;  $^{\circ}P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells. D. ELISA detection of nitrotyrosines in plasma membrane (PM)-derived extracts, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox/NODox-treated cells vs. Ctrl cells;  $^{\circ}P \leq 0.001$ : NODox-treated cells vs. corresponding Dox-treated cells. E. Expression of P-gp in ER-derived extracts. The CRT expression was used as control for equal protein loading. The figure is representative of 3 independent experiments. F. Expression of P-gp in plasma membrane-derived extracts. The pancadherin expression was used as control for equal protein loading. The figure is representative of 3 independent experiments. G. ELISA detection of nitrotyrosines in U-2OS/DX580 whole cell extracts, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox-treated cells vs. Ctrl cells;  $^{\circ}P \leq 0.001$ : NODox-treated cells vs. Dox-treated cells; # $P \leq 0.001$ : PT + NODox-treated cells vs. NODox-treated cells. H. Expression of P-gp in whole cell extracts of U-2OS/DX580 cells. The  $\beta$ -tubulin expression was used as control for equal protein loading. The figure is representative of 3 independent experiments.

plasma membrane.

We thus hypothesized that NODox-treated U-2OS/DX580 cells were functionally equivalent to U-2OS cells in terms of ICD. In sensitive cells, both Dox and NODox increased the DC-phagocytosis of tumor cells (Fig. 3D) and expanded the activated (i.e. CD107<sup>+</sup>IFN- $\gamma$ <sup>+</sup>) CD8<sup>+</sup> T-lymphocytes (Fig. 3E). In U-

2OS/DX580 cells only NODox, but not Dox, increased phagocytosis and activity of cytotoxic T-lymphocytes (Fig. 3D-E), suggesting a full restoration of ICD in these resistant cells.

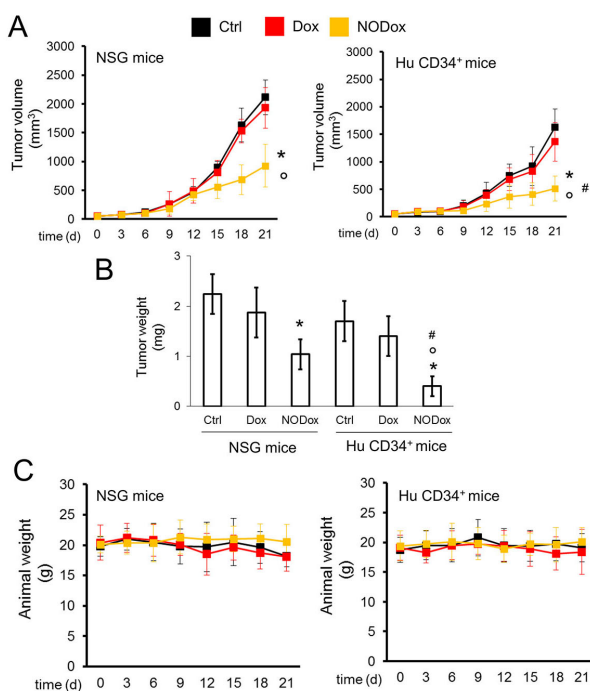
The anti-tumor efficacy of NODox was finally validated in U-2OS/DX580 xenografts, where Dox was completely ineffective (Fig. 4A). In both immune-deficient (left panel) and immune-



**Fig. 3. NO-releasing doxorubicin induces P-gp ubiquitination and restores the immunogenic cell death in human osteosarcoma cells.** Human Dox-sensitive U-2OS cells and Dox-resistant U-2OS/DX580 cells were incubated for 24 h in fresh medium (Ctrl) or in medium containing 5  $\mu$ M Dox or 5  $\mu$ M NODox. When indicated, the NO scavenger PTIO (100  $\mu$ M, PT) was co-incubated. **A.** Whole cell extracts were immune-precipitated (IP) for P-gp, then immunoblotted (IB) for mono/poly-ubiquitin (UQ). The  $\beta$ -tubulin expression was used as control for equal protein loading, before immunoprecipitation. No Ab: U-2OS/DX580 cells treated with NODox, immunoprecipitated in the absence of anti-Pgp antibody, as control of specificity. The figure is representative of 3 independent experiments. **B.** The ATPase activity of immune-purified P-gp, extracted from U-2OS/DX580 cells, was measured spectrophotometrically, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox-treated cells vs. Ctrl cells; ° $P \leq 0.001$ : NODox-treated cells vs. Dox-treated cells; # $P \leq 0.001$ : PT + NODox-treated cells vs. NODox-treated cells. **C.** Expression of CRT in plasma membrane-derived extracts (surface CRT). The same extracts were immunoprecipitated (IP) for CRT and immunoblotted (IB) for P-gp. The pancadherin expression was used as control for equal protein loading, before immunoprecipitation. CRT<sup>+</sup>: U-2OS/DX580 cells constitutively over-expressing CRT, used as positive control of CRT/P-gp-expressing cells. The figure is representative of 3 independent experiments. **D.** Tumor cells were stained with PKH2-FITC, DCs were stained with an anti-PE-conjugated-HLA-DR antibody. Tumor cells were co-incubated with DCs for 24 h. Double stained cells were counted by flow cytometry, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox-treated cells vs. Ctrl cells; ° $P \leq 0.001$ : NODox-treated cells vs. Dox-treated cells. **E.** T-lymphocytes were co-cultured for 10 days with DCs after phagocytosis, collected and co-cultured for 24 h with U-2OS and U-2OS/DX580 cells, treated as indicated previously. The percentage of CD8<sup>+</sup>CD107<sup>+</sup>INF- $\gamma$ <sup>+</sup> T-cells was measured by flow cytometry, in duplicates. Data are means  $\pm$  SD (n = 3 independent experiments). \* $P \leq 0.001$ : Dox-treated cells vs. Ctrl cells; ° $P \leq 0.001$ : NODox-treated cells vs. Dox-treated cells.

competent (right panel) mice, NODox reduced osteosarcoma growth. The rate of tumor growth was lower in immune-competent animals, in agreement with previous findings [33], suggesting that the host immune system is important in counteracting osteosarcoma growth. Notwithstanding the lower aggressiveness, Dox was unable to reduce tumor growth in immune-competent mice. By contrast, NODox was more effective in immune-competent mice than in immune-deficient ones (Fig. 4A), suggesting that part of Dox cytotoxicity is due to the engagement of the host immune system against the tumor. The reduction in tumor growth was con-

firmed by the weights of the excised tumors (Fig. 4B): in both immune-deficient and immune-competent mice NODox, but not Dox, significantly reduced tumor weight. The weights of tumors were slightly lower in immune-competent animals (Fig. 4B), in agreement with the different tumor growth rate (Fig. 4A). NO-Dox confirmed its maximal efficacy in immune-competent mice (Fig. 4B). The animal weight did not vary significantly between the group of treatments (Fig. 4C), suggesting that tumor-related cachexia or toxicities related to the treatments were unlikely in our experimental protocol.



**Fig. 4. NO-releasing doxorubicin reduces the growth of resistant osteosarcoma, particularly in immune-competent animals.** U-2OS/DX580 cells were s.c. implanted in 6-week old female NSG mice (left panel) or Hu-CD34<sup>+</sup> mice (right panel). When tumors reached a volume of 50 mm<sup>3</sup>, animals were randomized in 3 groups (n = 6 mice/group) and treated at day 3, 9, and 15 after randomization as it follows: 1) vehicle (Ctrl) group, treated with 0.1 ml saline solution i.v.; 2) Dox group, treated with 5 mg/kg Dox i.v.; 3) NODox group, treated with 5 mg/kg NODox i.v. A. Tumor growth was monitored daily by caliper measurement. Data are presented as means  $\pm$  SD. \* $P \leq 0.001$ : NODox group vs. Ctrl group (days 18-21); ° $P \leq 0.001$ : NODox group vs. Dox group (days 18-21); # $P \leq 0.022$ : Hu-CD34<sup>+</sup> mice vs. NSG mice, NODox group (days 18-21). B. Tumor weight after excision. Data are presented as means  $\pm$  SD. \* $P \leq 0.001$ : NODox group vs. Ctrl group; ° $P \leq 0.001$ : NODox group vs. Dox group; # $P \leq 0.043$ : Hu-CD34<sup>+</sup> mice vs. NSG mice, NODox group. C. Animal weight during treatment. Data are presented as means  $\pm$  SD.

## 4. Discussion

NO is a double edge-sword in cancer: at nanomolar concentrations it may have a pro-tumoral effect, at high micromolar concentrations it has anti-cancer activity [34]. The ability of NO to react with O<sub>2</sub><sup>-</sup> produces huge amounts of reactive nitrogen species (RNS) as ONOO<sup>-</sup>. This radical can exert cytotoxic effects or act as post-translational modifier of proteins, by inducing a stable tyrosine nitration or a labile cysteine nitrosylation [35]. Nitration and nitrosylation can increase or decrease protein activity, implying a fine modulation of signal transduction and catalytic functions [35]. Among the proteins nitrated, we previously identified several proteins of the ATP binding cassette (ABC) transporters family, implicated in the efflux of chemotherapeutic drugs. Following nitration, the catalytic cycle of the transporter is inhibited and chemotherapeutic drugs are more retained within cancer cells, increasing their cytotoxicity [36, 24]. Besides NO donors [36], ABC transporters' nitration can be elicited by synthetic NO-releasing

Dox that deliver both the NO donor inhibiting the ABC transporter and the chemotherapeutic drug within the tumor cell. These synthetic Dox [25, 37] and their liposomal formulations [27, 38] have successfully overcome the resistance to Dox in P-gp-expressing tumors, including osteosarcoma [22]. Higher is the intracellular retention of Dox, higher is the direct killing of cancer cells and the ability of triggering ICD [30]. In the present work, carried out on osteosarcoma cells with increasing levels of P-gp, we confirmed that NODox maintained the cytotoxic potential, in terms of apoptosis induction, reduced cell viability and ICD induction, also in P-gp-expressing cell, where Dox was ineffective. Both NO or its downstream mediator cyclic guanosine 3',5'-monophosphate (cGMP) [34] can mediate such chemosensitization. Indeed, it has been shown that the type 5 cGMP-phosphodiesterase inhibitor Sildenafil, which increases the intracellular levels of cGMP, increases the apoptosis mediated by Dox in drug-resistant prostate cancer cells as well [39]. Also, Sildenafil directly impaired the efflux activity of P-gp, reversing the resistance to paclitaxel in P-gp-over-expressing cells [40]. Although we did not investigate if cGMP was responsible for the reversion of resistance to Dox in osteosarcoma, our findings were in line with these previous works, because we clearly demonstrated that the release of NO, which can increase cGMP, from the synthetic NODox overcomes the resistance to parental Dox.

The first reason for the different effect of Dox and NODox was the intracellular retention of the two drugs. The amount of Dox retained within P-gp-expressing cells was likely insufficient to elicit any direct cytotoxic effect nor to induce the ER stress that primes cells for ICD [41]. By contrast, NODox accumulation in U-2OS/DX580 cells was comparable to the accumulation of Dox in U-2OS cells, which underwent a classical ICD.

Second, one of the mechanism triggering CRT translocation and ICD is the increase of NO that activates the guanylate cyclase/PKG axis, induces a cytoskeleton rearrangement and promotes the translocation of CRT to the plasma membrane [19]. In addition, NO itself is an inducer of ER stress [42]: the nitrosative stress caused by NO up-regulates the ER stress sensor C/EBP- $\beta$  LIP [43], which decreases P-gp [44] and increases CRT [29]. These events trigger a virtuous circle that amplifies Dox cytotoxicity, by decreasing the expression of its main efflux transporter and favoring the exposure of CRT. In our experimental conditions, Dox (in U-2OS cells) and NODox (in U-2OS and U-2OS/DX580 cells) elicited a nitrosative stress, as indicated by the increasing amount of nitrotyrosines in whole cells and in the key compartments -- ER and plasma membrane -- where P-gp is synthesized and active. P-gp was nitrated in all these compartments, although the mechanisms inducing nitration were likely different. Dox does not release NO, but it induces the production of micromolar amounts of NO by inducing NOS2 [24, 36, 42]. The induction of NOS2 is dose-dependent: it happens in Dox-sensitive cells, not in Dox-resistant ones, where P-gp effluxes the drug [24, 36]. This mechanism explains why Dox increased nitrotyrosines in U-2OS cells, not in U-2OS/DX580 cells. By contrast, NODox releases NO once entered the cells: although the intracellular localization of NODox is mainly mitochondrial [37], NO can diffuse intracellularly and react with O<sub>2</sub><sup>-</sup>. The ONOO<sup>-</sup> produced has a diffusion range of few microns [45] that is sufficient to elicit nitration of proteins in dif-

ferent cellular compartments. The rate of nitration depends on the amount of  $\text{ONOO}^-$ , on the amount of the target proteins, on the amount and exposure of tyrosines within the target proteins [46]. While the amount of tyrosines nitrated by NODox was the same in U-2OS and U-2OS/DX580 cells, suggesting a comparable level of NO released and  $\text{ONOO}^-$  generated, P-gp resulted nitrated in U-2OD/DX580 cells only. The absence of P-gp in U-2OS cells and the abundance of the protein in U-2OS/DX580 cells may explain while we detected P-gp nitrated in the resistant variant only. In line with previous findings [43], the nitration determined a strong reduction in the catalytic activity.

Interestingly, in U-2OS/DX580 cells treated with NODox, we noticed a reduction in the amount of P-gp in whole cell lysates, as well as in ER and plasma membrane extracts. Since nitration is accompanied by protein oxidation and unfolding [47], it can destabilize the target protein and prime it for ubiquitination. This mechanism was elicited by NODox that induced a strong NO-dependent poly-ubiquitination of P-gp, explaining the lower amount of protein detected in all cell compartments. A band of 170 kDa, likely corresponding to mono-ubiquitinated P-gp, was detected in U-2OS/DX580 cells. Mono-ubiquitination often promotes the endocytosis of membrane proteins within lysosomes [48]. In the case of P-gp, the lysosomal localization contributes to drug resistance, because the P-gp present on the lysosomal membrane is active and sequesters Dox within lysosomes [49]. Therefore, it is not surprising to find a constitutively mono-ubiquitinated P-gp in the highly resistant osteosarcoma cells. By contrast, poly-ubiquitination typically primes proteins for proteasomal degradation [50].

The removal of P-gp from cell surface positively impacted on the induction of ICD, since P-gp interacts with CRT, masks its immune-sensitizing functions and impairs the recognition of tumor cells by DCs [19, 20]. P-gp-expressing cells are chemo- and immuno-resistant not only because of low accumulation of Dox and low production of NO, but also because of defective functions of CRT. Thanks to the reduction of P-gp amount and to the increased production of NO, NODox increased the immune-sensitizing functions of CRT that is free to translocate on the surface of resistant cells, without suffering any inhibition by P-gp. As a consequence, P-gp-expressing U-2OS/DX580 cells treated with NODox have the same sensitivity to ICD of P-gp-negative U-2OS cells, being phagocytized by DCs and inducing a significant increase of activated  $\text{CD8}^+$  T-lymphocytes.

The efficacy of NODox and the involvement of the immune system in the cell death induced by the drug was also validated in U-2OS/DX580 xenografts that were completely refractory to Dox. NODox significantly reduced tumor growth in immune-deficient mice, but it had a more pronounced effect in immune-competent humanized mice. A competent immune system is important in controlling osteosarcoma progression, as demonstrated by the lower growth rate of U-2OS/DX580 xenografts in humanized mice. In these models, NODox exerted a stronger anti-tumor activity likely by activating the host immune system against the tumor. The critical role of a competent immune system against osteosarcoma is in agreement with a previous work showing that the activation of DCs by tumor lysates and the administration of anti-CTL4 antibody, which relieves the anergy of cytotoxic T-lymphocytes, slow down the progression of osteosarcoma [51].

## Conclusions

We identified NODox as a drug with pleiotropic effects on P-gp and as a potentially effective tool agent against Dox-resistant osteosarcomas. Although it is well-known that NO donors are both chemo- and immune-sensitizers [52], our approach is innovative because it conjugates the advantage of using Dox, already employed as first-line treatment in osteosarcoma, with the advantage of using NO, an inhibitor of expression and activity of P-gp. These two events enforce the direct killing and the ICD induced by Dox, transforming chemo-immune-resistant osteosarcoma cells into chemo-immune-sensitive ones. Compared to parental Dox, NODox has also a safer profile of cardiovascular toxicity [27, 53] that constitutes an additional advantage. Together, these evidences suggest the possibility to start clinical trials based on NODox in patients with Dox-resistant, P-gp-expressing osteosarcomas, characterized by a poor prognosis.

## Author contributions

CC and JK performed the *in vitro* experiments; JK performed the assays in xenograft models; CC and JK analyzed the data; CR supervised the study and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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## Conflict of interest statement

The authors declare no competing interests.

## Abbreviations

Dox, doxorubicin; P-gp: P-glycoprotein; DC, dendritic cell; ICD, immunogenic cell death; ER: endoplasmic reticulum; CRT: calreticulin; NO, nitric oxide; NODox, nitric oxide-releasing doxorubicin; DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanine; s.c., subcutaneously; NSG, NOD SCID gamma; i.v., intravenously; ANOVA, analysis of variance; SPSS; Statistical Package for Social Science; NOS2, NO synthase 2; ABC, ATP binding cassette; cGMP: cyclic guanosine 3',5'-monophosphate.

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