Research article

Novel anthracyclines with enhanced immunogenic effects against drug resistant osteosarcoma cells

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Abstract

Doxorubicin (dox) is one of the first-line drug treatment in osteosarcoma. P-glycoprotein (Pgp) limits dox’s intracellular accumulation and efficacy in osteosarcoma. Part of the cytotoxic effects of dox are mediated by the induction of immunogenic cell death (ICD) that allows a durable eradication of the tumor by the host immune system. Pgp-overexpressing tumors, however, are also ICD-resistant. We recently synthesized two classes of synthetic doxs – nitric oxide (NO)-releasing dox and H₂S-releasing dox – that were cytotoxic against different Pgp-expressing tumors. The aim of this work is to investigate if the lead compounds (termed Ndox and Sdox) were able to elicit ICD in Pgp-positive/dox-resistant osteosarcoma cells. Ndox and Sdox induced apoptosis in both sensitive and resistant cells, were localized within the endoplasmic reticulum (ER), up-regulated ER stress-dependent cell death genes, promoted the translocation of calreticulin form ER to cell surface, induced the extracellular release of ATP and HMGB1, increased the phagocytosis of tumor cells by dendritic cells and the expansion of anti-tumor CD8+ T-lymphocytes, in a NO- and H₂S-dependent manner, respectively. Expanded CD8+ clones up-regulated immun-activating cytokines and down-regulated immune-suppressive cytokines. Dox induced the same events in sensitive cells, but not in Pgp-expressing/dox-resistant cells. We suggest Ndox and Sdox as new multifunctional anthracyclines able to induce apoptosis of resistant osteosarcoma cells and contemporarily activate an anti-tumor immune response. These pro-drugs may have a future use in osteosarcoma patients with high Pgp expression, characterized by a poor outcome because of the lack of durable tumor eradication and the high frequency of relapse.

Keywords

P-glycoprotein; Osteosarcoma; Doxorubicin; Endoplasmic reticulum stress; Immunogenic cell death

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Abbreviations

Dox, doxorubicin; Pgp, P-glycoprotein; DC, dendritic cell; CAR, chimeric antigen receptor; ICD, immunogenic cell death; CRT, calreticulin; ER, endoplasmic reticulum; NO, nitric oxide; Ndox, nitric oxide-releasing doxorubicin; Sdox, H₂S-releasing doxorubicin; FBS, fetal bovine serum; MRPI, multidrug resistance related protein 1; BCRP, breast cancer resistance protein; FITC, fluorescein isothyocianate; PI, propidium iodide; GFP, green fluorescence protein; PBS, phosphate buffer saline; HMGB1, High Mobility Group Protein 1; PE, phycoerythrin; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance; carboxy-PTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; DAMP, danger-associated molecule pathway; CTA, cancer/testis antigen; UPR, unfolded protein response.

1. Introduction

Osteosarcoma is the most frequent tumor in the bone. The standard therapy is based on neo-adjuvant and adjuvant polychemotherapy, including doxorubicin (dox), cisplatin and methotrexate. Complete or partial responses are achieved no more than 60% patients [1, 2]. The main reason of dox’s failure is the presence of ABCB1/P-glycoprotein (Pgp) that limits intracellular dox’s accumulation and cytotoxicity [3], and is a negative prognostic factor [4].

Recently, immune-therapy-based approaches have been evaluated to improve the patients’ outcome. Osteosarcoma grows faster in immune-deficient mice than in immune-competent mice [5], suggesting that an active immune-surveillance may control the disease. Increasing the number of activated cytotoxic CD8+ T-lymphocytes [6], activating dendritic cells (DCs) to process osteosarcoma antigens and expand anti-tumor CD8+ T-cells [7, 8], employing immunogenic oncolytic viruses [9], macrophage-activating drugs such as mifamurtide [10], chimeric antigen receptor (CAR) T-cells [11], immune-checkpoint inhibitors such as anti-PD-1 or anti-CTLA-4 [12], are under evaluation in preclinical models and/or in clinical trials. Until now, combined chemo-immune-therapy has obtained controversial results. For instance, standard chemotherapy followed by the infusion of autologous T-lymphocytes or DCs activated by tumor lysates has increased patients survival [8]. Trabectedin has reduced osteosarcoma growth and metastasis in immune-competent mice, increased cell differentiation and CD8+ T-lymphocyte recruitment within the tumor. However, these T-cells have high expression of PD-1 and exhausted phenotype [13].

Most studies on chemo-immune-therapy regimes did not consider the Pgp expression in osteosarcoma cells. Indeed, dox is a strong activator of immunogenic cell death (ICD), i.e. the tumor
Fig. 1. NO-and H₂S-releasing doxorubicins are accumulated and induce apoptosis in doxorubicin-resistant osteosarcoma cells. A. Expression of Pgp, MRP1 and BCRP in human doxorubicin-sensitive U-2OS cells and in their doxorubicin-resistant variants DX30, DX100, DX580, evaluated by immunoblotting. The β-tubulin expression was used as control of equal protein loading. The figure is representative of 1 out of 3 experiments. B. Intracellular doxorubicin accumulation, measured after a 6 h incubation with 5 µM doxorubicin (dox), NO-releasing doxorubicin (Ndox) or H₂S-releasing doxorubicin (Sdox), by a fluorimetric assay, in duplicates. Data are means ± SD (n = 4 independent experiments). *p < 0.002 for DX-cells vs. parental U-2OS cells; ◦ p < 0.001 for Ndox/Sdox vs. dox. C. Cell were incubated 24 h with drug-free medium (ctrl), 5 µM dox, Ndox or Sdox. The percentage of cells positively stained for Annexin V-FITC or propidium iodide (PI) was measured by flow cytometry, in duplicates. Dot plots are representative of 1 out of 3 experiments.

cell killing followed by the recruitment of local DCs, DC-mediated phagocytosis and activation of anti-tumor CD8⁺T-lymphocytes [14], but it loses the immunogenic properties in Pgp-expressing tumors. First, by pumping out dox, Pgp limits the intracellular dox’s accumulation and does not allow to reach concentrations necessary to induce ICD [15]. Second, Pgp inhibits the immunogenic function of calreticulin (CRT), a protein translocated from endoplasmic reticulum (ER) to plasma-membrane upon ER stress or dox treatment, and necessary to the recruitment of DC [16].

In this work, we investigated two new synthetic doxs as potential chemo-immune-therapy agents, i.e. compounds able to induce cytotoxicity and ICD in Pgp-expressing osteosarcoma cells. The first synthetic dox investigated is a nitric oxide (NO)-releasing dox (Ndox), which is effectively retained within Pgp-expressing cells thanks to the release of NO, a non-covalent inhibitor of Pgp [17]. Of note, NO promotes ER stress [18] and translocation of CRT [16]. For these reasons, we hypothesized that Ndox may have a superior immunogenic potential compared to dox. The second synthetic dox tested is a H₂S-releasing dox (Sdox), which is cytotoxic against Pgp-positive/dox-resistant osteosarcoma cells [19]. Interestingly, Sdox induces ER stress [20], producing a condition that should favor ICD.

We demonstrated that Ndox and Sdox effectively elicited ICD and promoted the DC-mediated activation of anti-tumor CD8⁺T-lymphocytes in Pgp-positive osteosarcoma cells, resistant to parental dox.

2. Materials and Methods
2.1. Chemicals

Fetal bovine serum (FBS) and culture medium were from Invitrogen Life Technologies (Carlsbad, CA). Plastic ware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). Dox was purchased by Sigma Chemical Co (St.
Louis, MO). Unless otherwise specified, all the other reagents were purchased from Sigma Chemical Co.

2.2. Drugs synthesis

Ndox and Sdox were synthesized as described in [17] and [19], respectively: Ndox corresponds to compound 4 in [17]; Sdox corresponds to compound 10 in [19]. The chemical structures of dox, Ndox and Sdox are reported in the Supplementary Fig. 1.

2.3. Cell lines

Human dox-sensitive osteosarcoma U-2OS cell line was purchased from ATCC (Manassas, VA). The corresponding variants with increasing resistance to dox (U-2OS/DX30, U-2OS/DX100, U-2OS/DX580), selected by culturing parental cells in medium containing progressively increased dox dosages - 30, 100, 580 ng/mL dox, respectively - were generated as reported in [21]. All cell lines were authenticated by microsatellite analysis, using the PowerPlex kit (Promega Corporation, Madison, WI; last authentication: December 2018). Cells were maintained in medium supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin, 1% v/v L-glutamine.

2.4. Immunoblotting

Cells were rinsed with ice-cold lysis buffer (50 mM Tris, 10 mM EDTA, 1% v/v Triton-X100; pH 7.5), supplemented with the protease inhibitor cocktail set III (80 μM aprotinin, 5 mM bestatin, 1.5 mM leupeptin, 1 mM pepstatin; Calbiochem, San Diego, CA), 2 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4. Cells were sonicated (10 bursts of 10 sec, 4°C), washed with Tris-buffered saline-Tween 0.1% v/v and centrifuged at 13,000 x g for 10 min at 4°C. 20 μg of protein extracts were subjected to 4-20% gradient electrophoresis and probed with the following antibodies: anti-ABCBI/Pgp (1: 500, Calbiochem); anti-ABCC1/multidrug resistance related protein 1 (MRP1; 1: 200, Abcam, Cambridge, UK); anti-ABC2/breast cancer resistance protein (BCRP; 1: 500, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-β-tubulin (1: 1,000, Santa Cruz Biotechnology Inc.). The membranes were incubated with peroxidase-conjugated secondary antibodies (1: 3,000, Bio-Rad Laboratories) and washed with Tris-buffered saline-Tween 0.1% v/v solutions. Protein bands were detected by enhanced chemiluminescence (Bio-Rad Laboratories).

2.5. Doxorubicin accumulation

Dox content was measured fluorimetrically as detailed previously [22], using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Winoosky, MT). The results were expressed as nmoles dox/mg cell proteins.

2.6. Cell apoptosis

1 x 10⁶ cells were stained with the Apoptosis Detection kit (Sigma Chemicals Co.), using either Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), as per manufacturer’s instructions. Samples were analyzed using a Guava® easyCyte flow cytometer (Millipore, Bedford, MA), equipped with the InCyte software (Millipore).

2.7. Intracellular drug localization

5 x 10⁵ cells were grown on sterile glass coverslips and transfected with the green fluorescence protein (GFP)-KDEL fused-CRT expression vector (Cell Light BacMan 2.0, Invitrogen Life Technologies) to label ER. After 24 h, cells were incubated with 5 μM dox, Ndox or Sdox for 6 h. Samples were rinsed with phosphate buffer saline (PBS), fixed with 4% v/v paraformaldehyde for 15 min, washed three times with PBS and once with water, mounted with 4 μl of Gel Mount Aqueous Mounting. Slides were analysed using Leica DC100 fluorescence microscope (Leica, Wetzlar, Germany). For each experimental condition, a minimum of 5 microscopic fields were examined.

2.8. PCR arrays

Total RNA from osteosarcoma cells or T-lymphocytes was extracted and reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). The PCR arrays were performed on 1 μg cDNA, using the RT² Profiler Unfolded Protein Response Plus PCR Array, and the RT² Profiler Cytokine and Chemokine PCR Array (Bio-Rad Laboratories), as per manufacturer’s instructions. Data analysis was performed with the PrimePCR™ Analysis Software (Bio-Rad Laboratories).

2.9. ICD analysis

To measure the levels of surface CRT, 1 x 10⁵ cells were washed with PBS, detached with Cell Dissociation Solution (Sigma Chemicals Co.), incubated for 45 min at 4°C with an anti-CRT antibody (Affinity Bioreagents, Rockford, IL; 1: 100), followed by the AlexaFluor488-conjugated secondary antibody (Abcam; 1: 50) for 30 min at 4°C. After the fixation step in 2.5% v/v paraformaldehyde for 5 min at room temperature, samples were analyzed with a Guava® EasyCyte flow cytometer equipped with the InCyte software. Cells incubated with not-immune isotype antibody, followed by secondary antibody, were included as control of specificity. The ATP release was measured on 100 μl of the cell culture medium with the ATP Bioluminescent Assay Kit (FL-AA, Sigma Aldrich Co.), using a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments). The results were expressed as nmoles/mg cell proteins. The release of High Mobility Group Protein 1 (HMGB1) was measured using the High Mobility Group Protein 1 ELISA kit (Cloud-Clone Corp., Houston, Texas), as per manufacturer’s instructions. The results were expressed in pg/mg cell proteins.

2.10. Tumor cells phagocytosis and T-lymphocytes activation

DCs were generated from monocytes immuno-magnetically isolated from peripheral blood of healthy donors, provided by the Blood Bank of AOU Città della Salute e della Scienza, Torino, Italy, as previously reported [15]. The phagocytosis assay was performed as detailed in [15], by co-incubating DCs and tumor cells at 37°C or 4°C for 24 h. The percentage of phagocytized cells obtained after the incubation at 4°C was subtracted from the percentage obtained at 37°C, and was always less than 5% (not shown). The phagocytosis rate was expressed as phagocytic index [23]. After cell phagocytosis, DCs were washed and co-cultured for 10 days with autologous T-lymphocytes, isolated from peripheral blood by immuno-magnetic sorting using the Pan T Cell Isolation Kit (Miltenyi Biotec., Tetrov, Germany).
Germany). After the incubation with DC, T-lymphocytes were collected and co-cultured with tumor cells for 24 h. The expression of CD107, a degranulation marker and an index of active cytotoxic CD8+ T-lymphocytes, was determined by flow cytometry as previously reported [15], using FITC-conjugated anti-CD107 (1:10) and phycoerythrin (PE)-conjugated anti-CD107 (1:10) antibodies (Milenyi Biotec.). Samples were analyzed with a Guava® EasyCyte flow cytometer equipped with the InCyte software. The production of IFN-γ in the supernatant of CD8+ T-lymphocytes co-cultured with DC - a second parameter of CD8+ T-cells cytotoxic activity [24] - was measured with the Human IFN-γ DuoSet Development Kit (R&D Systems, Minneapolis, MN). The results were expressed as pg/mL.

2.11. Nitrite and H2S production

The production of nitrite, the stable derivative of NO, was measured spectrophotometrically by the Griess methods, as described in [25]. The nitrite concentration was expressed as nanomoles/mg cell proteins. The release of H2S was assessed by UV/high pressure liquid chromatography (HPLC), as detailed previously [17]. The results were expressed as nanomoles/mg cell proteins.

2.12. Statistical analysis

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

3. Results

3.1. NO-and H2S-releasing doxorubicins induce apoptosis and endoplasmic reticulum stress in osteosarcoma cells refractory to doxorubicin

We measured the intracellular retention of dox, Ndox and Sdox in U-2OS cells and in DX30, DX100, DX580 variants, that have progressively increased levels of Pgp, low levels of MRP1 and undetectable levels of BCRP in immunoblotting (Fig. 1A). While the intracellular amount of dox progressively decreased in the resistant variants, the retention of Ndox and Sdox remained significantly higher in all the cell lines. A slight - but not significant - decrease in the accumulation of Ndox and Sdox was observed in DX580 cells only (Fig. 1B). Consistently, dox induced necro-apoptosis - measured as percentage of annexin V-FITC/PI-positive cells - in U-2OS cells, but it progressively diminished its cytotoxicity in DX30, DX100 and DX580 variants. By contrast, Ndox and Sdox induced a strong necro-apoptosis in sensitive and resistant cells (Fig. 1C).

The intracellular localization of the drugs was analyzed in U-2OS cells, since dox was undetectable in the resistant variants [20], in consequence of its low intracellular accumulation. As expected, dox had a nuclear localization. By contrast, Ndox and Sdox were localized within the ER (Fig. 2A).

To further investigate the cytotoxic properties of Ndox and Sdox, we focused on dox-sensitive U-2OS cells and on the most dox-resistant variant, i.e. DX580 cells.

One of the mechanisms of dox-induced cell death is the induction of ER stress, an event that occurs in sensitive but not in resistant cells [20, 26]. Consistently with these findings, dox up-regulated the gene expression of ER stress sensors and ER stress-effectors of cell death, down-regulated the expression of ER stress-effectors of cell survival in U-2OS cells (Fig. 2B). These effects were lost in DX580 cells. Ndox and Sdox increased the expression of ER stress sensors and effectors of cell death, and decreased the expression of effectors of cell survival in both U-2OS and DX580 cells. These events were dependent on the release of NO and H2S, respectively. Indeed, when cells were treated with Ndox and the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO), at a concentration preventing the increase of NO released by Ndox (Supplementary Fig. 2A), the changes in the expression of ER stress sensors and effectors were attenuated (Fig. 2B). Similarly, the effects of Sdox on gene expression were strongly decreased in U-2OS and DX580 cells co-incubated with hydroxycobalamin (Fig. 2B), a H2S scavenger used at a concentration abrogating the increases of H2S in Sdox-treated cells (Supplementary Fig. 2B). As expected, carboxy-PTIO had no effects on dox- and Sdox-treated cells, hydroxycobalamin had no effects on dox-and Ndox-treated cells (Fig. 2B), confirming their specificity as scavengers of the NO released from Ndox and of the H2S released from Sdox, respectively.

3.2. NO-and H2S-releasing doxorubicins elicit an immunogenic cell death and induce an anti-tumor activation of CD8+ T-lymphocytes

ICD mediated by ER stress is characterized by the exposure of the “eat me” signal CRT and by the extracellular release of signals of danger-associated molecule pathways (DAMPs), such as ATP and HMGB1 [27]. Dox induced the exposure of CRT (Fig. 3A) and the release of ATP (Fig. 3B) and HMGB1 (Fig. 3C) in U-2OS cells, not in DX580 cells. By contrasts, such responses were elicited by Ndox and Sdox in both dox-sensitive and dox-resistant cells (Fig. 3A-C). Carboxy-PTIO and hydroxycobalamin did not change the exposure of CRT, and the release of ATP and HMGB1 in U2OS and DX580 cells, either untreated or dox-treated (Supplementary Fig. 3A-C). By contrast, carboxy-PTIO and hydroxycobalamin reduced all these events in cells treated with Ndox and Sdox, respectively (Fig. 3A-C).

U-2OS cells treated with dox, Ndox and Sdox were phagocytized by DCs (Fig. 4A). In these experimental conditions, CD8+ T-lymphocytes co-cultured with DCs that have phagocytized tumor cells, were endowed with cytotoxic activity, as demonstrated by the increased expression of CD107 (Fig. 4B), by the increased production of IFN-γ (Fig. 4C), by the up-regulation of immune-activating cytokines coupled with the down-regulation of immune-suppressive cytokines (Fig. 4D). In DX580 cells, Ndox and Sdox triggered the phagocytosis at the same extent of U-2OS cells, while dox was ineffective (Fig. 4A). Similarly, the treatment of DX580 with Ndox and Sdox - but not with dox - increased the percentage of CD8+ T-lymphocytes expressing CD107 (Fig. 4B), producing IFN-γ (Fig. 4C) and characterized by the high ratio immune-activating/immune-suppressive cytokines (Fig. 4D). Again, these events were mediated in both sensitive and resistant cells by the release of NO and H2S from Ndox and Sdox, as suggested by the complete abrogation of the increases in these immune-activation parameters elicited by carboxy-PTIO and hydroxycobalamin (Fig. 4A-D).

4. Discussion

In this work we describe the immunogenic effects of two synthetic anthracyclines, Ndox and Sdox, able to exert their cytotoxic potential against osteosarcoma cells resistant to parental dox. Notwith-
Fig. 2. NO-and H\textsubscript{2}S-releasing doxorubicins are localized within endoplasmic reticulum and up-regulate endoplasmic reticulum stress-related genes. 

A. U-2OS cells were incubated for 24 h with the GFP-KDEL-CRT expression vector to label endoplasmic reticulum (ER), then treated with 5 µM doxorubicin (dox), NO-releasing doxorubicin (Ndox) or H\textsubscript{2}S-releasing doxorubicin (Sdox) for the last 6 h. The intracellular localization of the drugs was analyzed by fluorescence microscopy. Magnification: 63 × objective lens (1.42 numerical aperture); 10 × ocular lens. Bar: 10 µm. The micrographs are representative of 3 experiments with similar results.

B. U-2OS and U-2OS/DX580 cells were treated 24 h treatment with drug-free medium, 5 µM doxorubicin (dox), NO-releasing doxorubicin (Ndox) or H\textsubscript{2}S-releasing doxorubicin (Sdox), in the absence or presence of the NO scavenger carboxy-PTIO (100 µM, P) or the H\textsubscript{2}S-scavenger hydroxycobalamin (100 µM, H). Heatmap of unfolded protein response (UPR)-related genes leading to ER stress, ER stress-related genes inducing cell death or cell survival, measured by PCR arrays in triplicates. The figure reports genes up-or down-regulated at least two-fold, in at least one cell line, compared to untreated U-2OS cells (n = 6 independent experiments). The expression of each gene in U-2OS cells was considered 1 (not shown).

Standing the presence of Pgp, Ndox and Sdox were well retained within the resistant cells, in consequence of two different mechanisms. Ndox is a strong releaser of NO that nitrates Pgp on tyrosines necessary for the catalytic cycle [15]. Sdox has a lower Km than dox for Pgp, suggesting that its affinity is lower than that of parental dox, and is effluxed with a lower Vmax, indicating that Sdox-treated cells also have a lower amount of Pgp [19]. Indeed, Sdox sulfhydrates nascent Pgp in the ER, disrupts specific disulfide bonds and induces the ubiquitination of unfolded Pgp [20]. These mechanisms allow Ndox and Sdox to reach concentrations sufficient to induce a significant apoptosis also in cells expressing Pgp, contrarily to dox.

Besides inhibiting drug efflux, NO and H\textsubscript{2}S can exert cytotoxic effects per se, justifying the apoptosis observed. Although the endogenously produced NO is tumorigenic in osteosarcoma [28], the release of high (i.e. micromolar) concentrations of NO – as in the case of Ndox – inhibits the tricarboxylic acid cycle and the electron transport chain, induces oxidative and nitrosative stress in mitochondria, triggers a caspase 9/caspase 3-dependent apoptosis in
different solid tumors [15, 29]. On the other hand, natural sulfide derivatives are highly cytotoxic for different cancer cells including osteosarcoma, where they reduce cell proliferation [30].

Higher is the intracellular accumulation of dox, higher is the ability of the drug to trigger not only apoptosis, but multiple mechanisms of cell death. One of these mechanisms is the induction of ER stress, i.e. a perturbation of proteins folding that is sensed by GRP78/BiP, IRE1α, EL2AK3/PERK and ATF4. This perturbation results in cell survival if the stress is short or in cell death if the stress persists [31]. High levels of GRP78 have been associated with dox- and cisplatin resistance in osteosarcoma [32], but ER stress is a documented pro-apoptotic mechanism in the same tumor where it activates Noxa and Puma [33]. These findings suggest that ER stress may determine a protective or damaging response in osteosarcoma, depending on the type and duration of stressing conditions. Dox activates the ER stress-dependent C/EBP-β LIP/CHOP/TRB3/caspase 3 axis in sensitive osteosarcoma cells, not in resistant ones [20]. The mechanism of the ER stress induction relies on the transient accumulation of dox within ER [20], where it exerts oxidative and nitrosative damages. This response is abrogated in resistant cells where dox is retained at a concentration too low to elicit ER stress. In the present work we observed that Ndox and Sdox were stronger inducers of ER stress than dox, thanks to their peculiar localization within the ER, and to the release of NO and H₂S. Indeed, the sequestration within ER subtracts Ndox and Sdox from the efflux of Pgp present on plasma-membrane, making these pro-drugs able to reach high intracellular concentrations, independently of the presence of Pgp. Moreover, the NO released from Ndox activates the C/EBP-β LIP/CHOP/TRB3/caspase 3 axis [18]; the H₂S released from Sdox sulfhydrates ER-nascent proteins and triggers a CHOP-dependent pro-apoptotic cascade [20]. These mechanisms explain why Ndox and Sdox up-regulated ER stress sensors and cell death effectors genes in both Pgp-negative/dox-sensitive and Pgp-positive/dox-resistant osteosarcoma cells. The attenuation of this gene expression profiling with specific scavengers of NO and
Fig. 4. NO-and H₂S-releasing doxorubicins elicit an anti-tumor immune response against doxorubicin-resistant osteosarcoma cells. U-2OS and U-2OS/DX580 cells were cultured for 24 h with drug-free medium (ctrl), 5 µM doxorubicin (dox), NO-releasing doxorubicin (Ndox) or H₂S-releasing doxorubicin (Sdox), in the absence or presence of the NO scavenger carboxy-PTIO (100 µM, P) or the H₂S-scavenger hydroxycobalamin (100 µM, H). A. Tumor cells were stained with PKH2-FITC, dendritic cells (DCs) were stained with an anti-HLA-DR-PE antibody. Tumor cells were co-incubated with DCs for 24 h. Double-stained cells were counted by flow cytometry, in duplicates. Data are presented as means ± SD (n = 6 independent experiments). *p < 0.001 for dox vs. ctrl; ◦ p < 0.001 for Ndox + P/Sdox + H vs. Ndox/Sdox; # p < 0.001 for Ndox/Sdox vs. dox.

B-C. 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. The percentage of CD8⁺ CD107⁺ T-cells was measured by flow cytometry (panel B), the production of IFN-γ in the supernatants was measured by ELISA (panel C), in duplicates. Data are presented as means ± SD (n = 6). *p < 0.002 for dox vs. ctrl; ◦ p < 0.001 for Ndox + P/Sdox + H vs. Ndox/Sdox; # p < 0.001 for Ndox/Sdox vs. dox.

D. Heatmap of immune-activating and immune-suppressive cytokines produced by T-lymphocytes, cultured as reported in B-C, measured by PCR arrays, in triplicates. The figure reports genes up- or down-regulated at least two-fold, in at least one cell line, compared to untreated U-2OS cells (n = 6 independent experiments). The expression of each gene in U-2OS cells was considered 1 (not shown).

H₂S suggests that the NO-and H₂S-released within the ER from the two pro-drugs are crucial players in inducing ER stress-dependent cell death.

ER stress is a condition favoring the exposure of CRT and the release of DAMPs like ATP and HMGB1, triggering ICD in cancer cells [27]. In line with the extent of ER stress induced, we found that dox elicited ICD in sensitive osteosarcoma cells only, Ndox and Sdox in both sensitive and resistant cells. It has been reported that
a high intratumor CRT is good prognostic factor in osteosarcoma patients [34] and that HMGB1 release is a marker of dox-induced necrosis [35]. Our work explains these findings, since we show that CRT and HMGB1 increase are premises to trigger ICD in osteosarcoma and are proofs of dox’s efficacy. The lack of ER stress and ICD in cells treated with Ndox and Sdox plus a NO/H₂S scavengers suggests that the NO-and H₂S-dependent ER stress is necessary to induce ICD. This hypothesis is supported by a previous study that identified ER stress inducers from the NCI-repository as strong ICD inducers in osteosarcoma [36]. Our approach is different because we induced ICD by using derivatives of dox, the first-line treatment in osteosarcoma, specifically modified in order to elicit ER stress. Moreover, our pro-drugs induced ICD also in resistant cells that are refractory to the ICD induced by dox [15, 37, 38].

To grant a durable eradication of tumor cells, ICD must be followed by an efficient DC-mediated phagocytosis and by the expansion of anti-tumor CD8⁺ T-lymphocytes. Different strategies have been employed to increase the frequency of DC and CD8⁺ T-lymphocytes against osteosarcoma, including anti-TGF-β antibodies [39], infusion of autologous DC exposed to tumor lysates, combined with dox [40] or anti-CTLA4 [7], epigenetic expression of cancer/testis antigens (CTAs) that expand T-lymphocytes clones directed against osteosarcoma cells [41]. Although these approaches are feasible and well-tolerated, they did not increase significantly the patients outcome [42]. None of these studies considered the expression of Pgp in the treated osteosarcomas. Since Pgp impairs the immune-recognition by DCs [16], it is possible that the lack of success of these strategies was due to the inclusion of dox-resistant/Pgp-positive osteosarcomas in the studies. Compared to the above mentioned approaches, Ndox and Sdox had the advantage to induce and efficient DC and CD8⁺ T-lymphocytes expansion also against Pgp-positive osteosarcoma cells.

Besides Pgp, several cytokines and chemokines present in the tumor immune-environment affect the immune-response. For instance, the immune-activating cytokine IL-2 increases the overall survival of mice bearing osteosarcoma, if combined with radiotherapy [43]. By contrast, IL-18 induces immune-suppression by decreasing the proliferation of effector CD4⁺ and CD8⁺ T-lymphocytes, and their production of IFN-γ, and by increasing the infiltration of myeloid-derived suppressor cells [44]. The immune-suppressive cytokine IL-8 induces Pgp transcription in osteosarcoma [45], generating a chemo- and immune-resistant environment. We detected the up-regulation of immune-activating cytokines in CD8⁺ T-lymphocytes co-cultured with DC after the phagocytosis of sensitive cells treated with dox. By contrast, an opposite scenario was detected in CD8⁺ T-lymphocytes co-cultured with DC after the phagocytosis of resistant cells. These data suggest that sensitive cells induce an immune-active/anti-tumor environment, while resistant cells produce immune-suppression in response to dox. By contrast, Ndox and Sdox maintained a high ratio immune-activating/immune-suppressive cytokines in CD8⁺ T-lymphocytes expanded after the phagocytosis of sensitive and resistant cells, preserving the ideal conditions for the cytotoxic activation of T-cells against the tumor. Also in this case the mechanism was dependent on the release of NO and H₂S within tumor cells, as demonstrated by the reversion of the cytokine expression profile elicited by the respective scavengers.

In summary, we propose two new anthracyclines that retain their ability to induce apoptosis, ICD and anti-tumor immune response against osteosarcoma cells, irrespectively from the resistance to parental dox. We are aware that our findings must be validated in preclinical models of dox-resistant osteosarcomas. If the results are confirmed, Ndox and Sdox may be considered noteworthy of investigation in patients with dox-resistant/Pgp-expressing osteosarcomas. These patients have a poor outcome and may have the greatest benefits from innovative treatments.

Authors’ contribution
EG, IB and CC performed the experiments on doxorubicin cytotoxicity and localization, and the PCR arrays, and analyzed the data; JK and BC performed the experiments on immunogenic cell death and ex vivo immune activation, and analyzed the data; CR supervised the study, wrote and revised the manuscript.

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Conflict of interest
The authors declare no conflict of interest.

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