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Hyaluronated liposomes containing H2S-releasing doxorubicin are effective against P-glycoprotein-positive/doxorubicin-resistant osteosarcoma cells and xenografts

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Cover Letter
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Torino, 4/12/2018

To the kind attention of
Prof. Manfred Schwab

Editor-in-Chief

Cancer Letters

Dear Professor Schwab,

we are pleased to submit our manuscript entitled "**Hyaluronated liposomes containing H2S-releasing doxorubicin are effective against P-glycoprotein-positive/doxorubicin-resistant osteosarcoma cells and xenografts**" as original article to Cancer Letters.

Doxorubicin (dox) is one of the first-line treatment in osteosarcoma but its success is limited by the presence of the efflux pump P-glycoprotein (Pgp) and by the onset of cardiotoxicity. We previously demonstrated that synthetic doxs conjugated with a H_2S-relasing moiety (Sdox) were less cardiotoxic and more effective than dox against Pgp-positive osteosarcoma cells *in vitro*. In order to increase the active delivery to tumor, we produced hyaluronic acid (HA) decorated liposomal formulations of Sdox (HA-Lsdox), exploiting the abundance of the HA receptor CD44 in osteosarcoma.

HA-Lsdox was more effective than dox and the FDA-approved liposomal dox Caelyx® against Pgp-positive osteosarcoma, displaying the same cardiotoxicity profile of Caelyx®. Differently from dox, HA-Lsdox delivered the drug within the endoplasmic reticulum (ER), where they induced protein sulphydration and ubiquitination, activated a ER stress pro-apoptotic response mediated by CHOP. HA-Lsdox also sulphydrated the nascent Pgp in the ER, reducing its activity.

We propose HA-Lsdox as an innovative tool noteworthy to be tested in Pgp-positive patients where the standard chemotherapy based on dox has a limited success.

The material is an original research. The manuscript has not been published previously and is not being considered concurrently by another journal.

Authors declare that there are no conflicts of interest.

Sincerely yours,

Chiara Riganti

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*Highlights (for review)

Highlights

Doxorubicin is the most important drug for first-line treatment of osteosarcoma

Doxorubicin resistance in osteosarcoma is mostly mediated by P-glycoprotein

Hyaluronated liposomes containing H_2S-releasing doxorubicin overcome resistance

Such formulations induce a ER-dependent cell death and inhibit P-glycoprotein

*Abstract
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Abstract

Doxorubicin (dox) is one leader drug in osteosarcoma treatment but its effectiveness is limited by the efflux pump P-glycoprotein (Pgp) and by the onset of cardiotoxicity. We previously demonstrated that synthetic doxs conjugated with a H_2S-relasing moiety (Sdox) were less cardiotoxic and more effective than dox against Pgp-overexpressing osteosarcoma cells. In order to increase the active delivery to tumor cells, we produced hyaluronic acid (HA) decorated liposomes containing Sdox (HA-Lsdox), exploiting the abundance of the HA receptor CD44 in osteosarcoma.

HA-Lsdox showed a favorable drug-release profile and had higher toxicity *in vitro* and *in vivo* than dox or the FDA-approved liposomal dox Caelyx® against Pgp-overexpressing osteosarcoma, displaying the same cardiotoxicity profile of Caelyx®. Differently from dox, HA-Lsdox delivered the drug within the endoplasmic reticulum (ER), inducing protein sulphydration and ubiquitination, and activating a ER stress pro-apoptotic response mediated by CHOP. HA-Lsdox also sulphydrated the nascent Pgp in the ER, reducing its activity.

We propose HA-Lsdox as an innovative tool noteworthy to be tested in Pgp-overexpressing patients, who are frequently less responsive to standard treatments in which dox is one of the most important drugs.

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Hyaluronated liposomes containing H2S-releasing doxorubicin are effective against P-glycoprotein-positive/doxorubicin-resistant osteosarcoma cells and xenografts

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Abstract

Doxorubicin (dox) is one leader drug in osteosarcoma treatment but its effectiveness is limited by the efflux pump P-glycoprotein (Pgp) and by the onset of cardiotoxicity. We previously demonstrated that synthetic doxs conjugated with a H_2S-relasing moiety (Sdox) were less cardiotoxic and more effective than dox against Pgp-overexpressing osteosarcoma cells. In order to increase the active delivery to tumor cells, we produced hyaluronic acid (HA) decorated liposomes containing Sdox (HA-Lsdox), exploiting the abundance of the HA receptor CD44 in osteosarcoma.

HA-Lsdox showed a favorable drug-release profile and had higher toxicity *in vitro* and *in vivo* than dox or the FDA-approved liposomal dox Caelyx® against Pgp-overexpressing osteosarcoma, displaying the same cardiotoxicity profile of Caelyx®. Differently from dox, HA-Lsdox delivered the drug within the endoplasmic reticulum (ER), inducing protein sulphydration and ubiquitination, and activating a ER stress pro-apoptotic response mediated by CHOP. HA-Lsdox also sulphydrated the nascent Pgp in the ER, reducing its activity.

We propose HA-Lsdox as an innovative tool noteworthy to be tested in Pgp-overexpressing patients, who are frequently less responsive to standard treatments in which dox is one of the most important drugs.

Keywords: liposomal doxorubicin; osteosarcoma; P-glycoprotein; endoplasmic reticulum stress; protein sulphydration

1. Introduction

Pre- and post-operative chemotherapy is the usual treatment for high grade osteosarcoma. The most widely used treatment for conventional high-grade osteosarcoma (tumor localized in the extremities, non-metastatic at clinical onset, arisen in patients younger than 40 years) is based on the surgical removal of primary tumor combined with systemic pre- and post-operative multidrug chemotherapy based on doxorubicin (dox), methotrexate and cisplatin [1-3]. Despite this aggressive multimodal approach, 35-40% of patients poorly respond to treatment and relapse and this behavior is frequently associated with the overexpression at diagnosis of the ATP binding cassette B1/P-glycoprotein (ABCB1/Pgp) which efflux dox [4,5] and is a negative prognostic factor in osteosarcoma patients [6-9]. A second reason is the onset of dox-related cardiotoxicity that limits the cumulative dose of the drug that can be administered [10]. The FDA-approved liposomal formulation of dox, Caelyx®, that significantly reduced the drug cardiotoxicity, was well-tolerated but it did not display an anti-tumor activity superior to dox in sarcoma [11,12], likely because it is not more effective than dox in Pgp-positive tumors [13,14].

Notwithstanding the multiple strategies adopted, the prognosis of patients with osteosarcoma has not significantly improved in the last years [3].

Recently, we synthesized a library of hydrogen sulfide (H_2S)-releasing doxorubicins that did not elicit any cardiotoxic effect but still retained their efficacy on Pgp-overexpressing osteosarcoma cells *in vitro* [15,16]. The lead compound, *i.e.* compound **10** in [15], here Sdox, was also effective against dox-resistant prostate tumor xenografts [17].

To improve the tumor-to-healthy tissues delivery ratio, increase Sdox stability and solubility, here we produced Sdox liposomal formulations decorated with hyaluronic acid (HA) moiety (HA-Lsdox). Of note, the HA-receptor CD44 is expressed in osteosarcoma [18], where it promotes tumor growth and metastatic dissemination [19-22]. CD44 silencing improves the response to dox and cisplatin [23], although the molecular mechanisms are completely unknown. The abundancy of CD44 in osteosarcoma makes it an ideal target to increase the delivery of the Sdox towards the

tumor. The use of a liposomal formulation similar to Caelyx® is expected to reduce dox cardiotoxicity. The use of Sdox instead of dox as cargo drug should guarantee good efficacy against dox-resistant/Pgp-overexpressing osteosarcoma and reduced cardiotoxicity as well. We exploited all these features to test the efficacy and safety, and to investigate the molecular mechanisms of anti-tumor effects of HA-Lsdox in preclinical models of dox-resistant osteosarcoma.

2. Material and methods

2.1. Materials and instruments

Sodium hyaluronate of MW 14,800 Da was purchased from Lifecore Biomedical (Chaska, MN). All the phospholipids were provided by Avanti Polar-Lipids distributed by Spectra 2000 (Rome, Italy). Cholesterol, dox and all the other chemicals were obtained from Sigma Chemicals Co. (St. Louis Mo). Plasticware for cell cultures was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Foetal bovine serum (FBS) and culture medium were supplied by Invitrogen Life Technologies (Carlsbad, CA). The synthesis of Sdox was performed as described previously [15]. Conjugate between 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and HA (HA-DPPE) was prepared using the method described in [24]. Reverse phase-high pressure liquid chromatography (RP-HPLC) analysis was performed as described in [15]. Differential scanning calorimetry (DSC) was performed using a Q200 DSC (TA Instruments, New Castle, DE).

2.2. Preparation of liposomes

Lsdox were prepared using the thin lipid film-hydration method mixing together chloroform solutions of 1,2-distearoil-*sn*-glycero-3-phosphocoline (DSPC), cholesterol (CHOL) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (mPEG2000-DSPE) in 75:19:6 molar ratio and Sdox (11% ratio mol drug/mol lipid). The mixture was then evaporated by rotary evaporator and dried under vacuum overnight. The resulting lipid film was hydrated with a 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulforic acid (HEPES) buffer (pH 7.4) and the suspension was vortex mixed for 10 min and bath sonicated. The formulations were then sequentially extruded (Extruder, Lipex, Vancouver, Canada) through 400 and then 200 nm

polycarbonate membrane (Costar, Corning Incorporated, NY) at a set temperature of 5 °C above the phase transition temperature of the lipid mixture. Liposomal preparations were purified from non-encapsulated Sdox through chromatography on Sepharose CL-4B columns, eluting with HEPES buffer at room temperature. Liposomes were stored at 4 °C.

To prepare HA-Lsdox, the same method of preparation was used and the lipid film was hydrated using a solution of HA-DPPE conjugate (3 molar ratio) in HEPES.

2.3. Liposomes characterization

The mean particle size and polydispersity index (PDI) of the liposomes were determined at 20 °C by Dynamic Light Scattering using a Zetasizer (Nano-ZS, Malvern instruments, UK). Size measurements were performed at a fixed angle of 173° after dilution of the liposome suspensions in MilliQ® water. The surface charge of liposomes was evaluated by zeta potential measurements after dilution of the suspensions in 10 mM KCl. Phospholipid phosphorous was assessed in each liposome preparation by phosphate assay after destruction with perchloric acid [25]. The amount of encapsulated Sdox was determined by RP-HPLC as previously reported [14,15].

Liposomal preparations were analyzed for physical stability in the storage conditions (4 °C) and for Sdox release in FBS and HEPES buffer at 37 °C as previously reported [26].

For DSC analysis about 15 mg of hydrated samples suspension samples were introduced into a 40 µl aluminium pan and analyzed. DSC runs were conducted from 25 °C to 80 °C at a rate of 5 °C/min under constant nitrogen stream (50 ml/min). The main transition temperature (Tm) was determined as the onset temperature of the highest peak.

2.3 Cells

Murine dox-resistant osteosarcoma K7M2 cells, human dox-sensitive osteosarcoma U-2OS were purchased from ATCC (Manassas, VA). The corresponding dox resistant variant U-2OS/DX580,selected by culturing parental cells in a medium with 580 ng/ml dox, were generated as reported in [27], and continuously cultured in presence of dox. Cells were maintained in DMEM supplemented with 10% v/v FBS, 1% v/v penicillin-streptomycin, 1% v/v L-glutamine.

2.4. Immunoblotting

20 μg of protein extracts were subjected to 4-20% gradient SDS-PAGE and probed with the following antibodies: anti-ABCB1/Pgp (Calbiochem); anti-CD44 (Abcam, Cambridge, UK); anti-CCAAT-enhancer-binding protein-β (C/EBP-β, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-C/EBP homologous protein/growth arrest and DNA damage 153 (CHOP/GADD153, Santa Cruz Biotechnology Inc.), anti-*Tribbles* homolog 3 (TRB3, Proteintech, Chicago, IL), anti-p53 up-regulated modulator of apoptosis (PUMA, Cell Signaling Technology, Danvers, MA); anti-caspase 12 (Abcam), anti-caspase 7 (Abcam), anti-caspase 3 (GeneTex, Hsinhu City, Taiwan), anti-β-tubulin (Santa Cruz Biotechnology Inc.), followed by peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA). Microsomal fractions were prepared using the Endoplasmic Reticulum Isolation Kit (Sigma Chemicals. Co), as per manufacturer's instructions. 100 μg of microsomal proteins probed with the anti-Pgp antibody or with an anti-calreticulin antibody (Affinity Bioreagents, Rockford, IL). To measure ubiquitinated Pgp, 50 μg of microsomal proteins were immunoprecipitaed with the anti-Pgp antibody, using 25 μl of PureProteome Magnetic Beads (Millipore, Billerica, MA), then probed an anti-mono/poly-ubiquitin antibody (Axxora, Lausanne, Switzerland).

2.5. Flow cytometry

1×10^6 cells were rinsed and fixed with 2% w/v paraformaldehyde (PFA) for 2 min, washed three times with PBS and stained with the anti-CD44 antibody (Abcam) for 1 h on ice, followed by an AlexaFluor 488-conjugated secondary antibody (Millipore) for 30 min. 1×10^5 cells were analyzed with EasyCyte Guava™ flow cytometer (Millipore), equipped with the InCyte software (Millipore). Control experiments included incubation with non-immune isotype antibody.

2.5 Intracellular and subcellular doxorubicin accumulation

Dox content in whole cell lysates, in nuclear and microsomal fractions - isolated with the Nuclear Extract kit (Active Motif, La Hulpe, Belgium) and the Endoplasmic Reticulum Isolation Kit (Sigma

Chemicals. Co), respectively - was measured fluorimetrically [28]. Subcellular localization of Sdox was measured by fluorescence microscope as reported [16].

2.6 Cytotoxity, apoptosis and cell viability

The extracellular release of lactate dehydrogenase (LDH), considered an index of cell damage and necrosis, was measured as reported in [28]. The activation of caspase 3, an index of apoptosis, was measured fluorimetrically as detailed in [29]. Cell viability was measured by the ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA), as per manufacturer's instructions.

2.7 In vivo tumor growth

1×10^7 K7M2 cells, re-suspended in 100 μl Matrigel, were subcutaneously implanted in 6-week old female Balb/C mice. Tumor volume was monitored by caliper and calculated according to the equation: equation $(LxW^2)/2$, where L=tumor length and W=tumor width. When tumor reached the volume of 50 mm^3, animals were randomized and treated as reported in Figure 2. Tumor volumes were monitored daily by caliper and animals were euthanized at day 48 after randomization with zolazepam (0.2 ml/kg) and xylazine (16 mg/kg). Tumors were collected and photographed, then homogenized for 30 s at 15 Hz, using a TissueLyser II device (Qiagen, Hilden, Germany) and clarified at 12,000 x g for 5 min. 10 μg of proteins from tumor lysates were used for the immunoblot analysis of CD44, Pgp and caspase 3, as reported above. The hematochemical parameters were measured on 0.5 ml of blood collected immediately after mice sacrifice, using the respective kits from Beckman Coulter Inc. (Miami, FL). Animal care and experimental procedures were approved by the Bio-Ethical Committee of the Italian Ministry of Health (#122/2015-PR).

2.8. PCR arrays

Total RNA was extracted and reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). The PCR arrays were performed on 1 μg cDNA, using the Unfolded Protein Response Plus PCR Array (Bio-Rad Laboratories), as per manufacturer's instructions. Data analysis was performed with PrimePCR™ Analysis Software (Bio-Rad Laboratories).

2.9. Protein sulphydration

The sulfhydration of microsomal proteins (100 μg) or immunopurified Pgp (50 μg) was measured according to [30].

2.10 Protein ubiquitination

The ubiquitination of microsomal proteins (100 μg) or immunopurified Pgp (50 μg) was measured with the E3Lite Customizable Ubiquitin Ligase kit (Life-Sensors Inc., Malvern, PA), as detailed in [16].

2.11. Pgp ATPase activity

The Pgp ATPase activity was measured in Pgp-rich membrane vesicles as described in [31].

2.12 Cell silencing

2×10^5 cells in 0.25 ml FBS/antibiotic-free medium were transfected either with non-targeting scrambled siRNA pools or siRNA pools targeting CHOP/GADD53 (target sequences: NM_001290183.1; NM_007837.4; ThermoScientific), as per manufacturer's protocol. The efficacy of silencing was verified by immunoblotting 48 h after the transfection.

2.12 Statistical analysis

All data in the text and figures are provided as means ± SD. The results were analysed by a one-way analysis of variance (ANOVA) and Tukey's test. $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Liposomes formulation and characterization

The efficacy of HA decorated liposomes encapsulating Sdox - a compound recently synthesized in our laboratory that showed impressive activity against dox-resistant tumor cells [15] - was evaluated against preclinical models of osteosarcoma, insensitive to dox and its liposomal formulation Caelyx®, overexpressing Pgp and positive for the HA receptor CD44.

Plain and HA liposomes encapsulating Sdox (Lsdox and HA-Lsdox) were prepared by hydration of the drug-lipid film followed by extrusion through polycarbonate filters to obtain homogenous small unilamellar vesicles. HA-Lsdox were prepared by adding the HA-DPPE conjugate during the hydration phase of lipid film: in this way the phospholipidic chain was incorporated into the

liposome membrane, while the HA was exposed towards the aqueous phase. Liposomes displayed a dimensional range from about 190 nm to 204 nm and the particle size tended to slightly increase in the HA-decorated formulations, the PDI was low for all the formulations (< 0.13), indicating a narrow and homogenous size distribution. The zeta potential value was negative and lower for HA-Lsdox compared to the Lsdox, due to the carboxylic negative residues of HA, confirming the presence of HA on their surface. The liposomes showed a good entrapment efficiency, which was similar for both formulations, indicating that the introduction of the HA-DPPE conjugate did not affect the Sdox encapsulation (**Table 1**). After 4 weeks of storage in HEPES buffer at 4 °C all the formulations still conserved 85% of the initial Sdox content and over this period and no appreciable size and/or zeta potential change and no precipitation or liposomes aggregation were observed. Lsdox and HA-Lsdox showed similar Sdox release profile in FCS and HEPES buffer at 37 °C: 50% of the compound was released after 72 h in buffer and after 48 h in serum. Moreover, it was observed that during the incubation at 37 °C Sdox encapsulated in liposomes was protected by rapid and undesired degradation process that had been studied in [15]; indeed, for encapsulated Sdox nor dox neither other degradation products were detected.

DSC analysis was performed to investigate the thermal changes caused by the incorporation of Sdox in the phospholipid bilayer (**Supplementary Fig. S1; Table S1**). The thermogram of pure DSPC showed the main transition peak at T_{onset} 54.2 °C. When Sdox was added, the main transition was shifted to lower temperatures (T_{onset} 52.3 °C) and broadening of melting temperature peaks was observed indicating a strong insertion of Sdox within the DSPC membrane. This phenomenon persisted in the presence of mPEG2000-DSPE and of HA-DPPE conjugate. Altogether these results indicate that we obtained liposomal formulations stable and able to improve Sdox solubility and stability in physiological media, in addition, the insertion of the targeting moiety HA did not influence the physico-chemical characteristics of the liposomes.

3.2. Hyaluronated liposomes containing H_2S-releasing doxorubicin are effective against doxorubicin-resistant osteosarcoma in vitro and in vivo

U-2OS and U-2OS/DX580 human osteosarcoma cell lines presenting, respectively, low and high P-gp expression levels, and murine K7M2 (**Fig. 1a**) are >50% positive for the HA receptor CD44 (**Fig. 1b**), with a significantly higher percentage of positivity for the Pgp-overexpressing cells. As expected, dox and Caelyx® had lower intracellular accumulation (**Fig. 1c**), induced lower cell damage (**Fig. 1d**), apoptosis (**Fig. 1e**) and higher viability (**Fig. 1f**) in U-2OS/DX580 and K7M2 cells. By contrast, Sdox, either as free drug or Lsdox was retained within Pgp-overexpressing U-2OS/DX580 and K7M2 cells (**Fig. 1c**) at a sufficient amount to induce a significant damage and apoptosis, and reduce viability (**Fig. 1d-f**). These effects were significantly amplified using HA-Lsdox (**Fig. 1c-f**). Neither Sdox nor its liposomal formulations increased the efficacy of dox or Caelyx® in dox-sensitive U-2OS cells.

In line with the absent efficacy *in vitro*, neither dox nor Caelyx® reduced the growth (**Fig.2a-b**) nor induce intratumor apoptosis (**Fig. 2c**) in K7M2 tumors, chosen as a prototypical example of CD44 and Pgp-overexpressing osteosarcoma (**Fig. 2c**). A progressively decrease in the rate tumor growth paralleled by a progressive increase in the activation of intra-tumor caspase 3 was elicited by Sdox formulations, following this rank order: Sdox<Lsdox<HA-Lsdox (**Fig.2a-c**). Interestingly, these agents also progressively reduced the intratumor expression of Pgp, contrarily to dox and Caelyx® (**Fig. 2c**). Since HA is highly abundant in extracellular matrix (ECM) of sarcomas, and in many tumors it serves as a reservoir of growth factor and nutrients allowing their controlled delivery to tumor cells [32], we exploited the presence of HA on the surface of Sdox-carrying liposomes, in order to enhance the homing of liposomes osteosarcoma ECM and the subsequent intratumor delivery of their cargo. Notwithstanding the increasing use of liposomes and nanoparticles in osteosarcoma treatments [33-38], we were the first ones to validate a liposome-based treatment effective against dox-resistant/Pgp-overexpressing tumors.

Sdox and the liposomal formulations did not display signs of liver and kidney toxicity, according to the hematochemical parameters of the treated animals. As expected, dox increased the cardiotoxic

parameters CPK, CPK-MB and cTNT, while Caelyx® did not. Notably, Sdox, Lsdox and HA-Lsdox had a cardiotoxicity profile superimposable to Caelyx® (**Supplementary Table S2**).

3.3. Hyaluronated liposomes containing H_2S-releasing doxorubicin localized within endoplasmic reticulum where they impair the ERAD/ERQC system

Dox is known to have a typical intranuclear localization [29], that was confirmed in the parental U-2OS cells with low Pgp levels (**Fig. 3a**) and was dramatically reduced in Pgp-overexpressing U-2OS/DX580 and K7M2 cells (**Fig. 3a**). Caelyx® followed the same trend. By contrast, Sdox and its liposomal formulations showed a very low accumulation within the nucleus (**Fig. 3a**), while must of dox was recovered within the microsomal fractions (**Fig. 3b; Supplementary Fig. S2**). The maximal delivery within ER was reached by HA-Lsdox and was in line with the higher accumulation in the whole cell. This trend can be due to the higher presence of CD44 on dox-resistant cells surface, a feature that may favor the receptor-driven uptake of Sdox, and/or to the decreased efflux of Sdox via Pgp [15,16].

A targeted expression profile of ER-associated degradation/endoplasmic reticulum quality control (ERAD/ERQC)-related genes – that play a critical role in the folding and export of nascent proteins from the ER [39,40] - revealed that – contrarily to dox and Caelyx® - Sdox and its liposomal formulations up-regulated most of ERAD/ERQ genes in Pgp-positive K7M2 cells (**Fig. 3c**). ER-associated proteins were significantly more sulphydrated in K7M2 cells exposed to Sdox, Lsdox and in particular HA-Lsdox (**Fig. 3d**). Such sulphydration was paralleled by an increased ubiquitination (**Fig. 3e**), suggesting that the up-regulation of ERAD/ERQC gene was a compensatory response to the ER stress induced by Sdox [16] but it was not sufficient to rescue a proper folding of ER-associated proteins. The ubiquitination was likely consequent to the sulphydration elicited by the H_2S release, as demonstrated by the abrogation of either sulphydration and ubiquitination obtained by the H_2S scavenger hydroxyl-cobalamin (**Fig. 3d-e**). In line with the low retention within the ER (**Fig. 3b**), the results of gene expression profile (**Fig. 3c**) and the

absence of groups releasing H_2S, dox and Caelyx® did not induce sulphydration nor ubiquitination of ER proteins (**Fig. 3d-e**).

3.4. Hyaluronated liposomes containing H_2S-releasing doxorubicin trigger a ER-dependent apoptosis in doxorubicin-resistant osteosarcoma cells

An increased burden of misfolded proteins within ER elicits an adaptive response known as unfolded protein response (UPR) that can successfully overcome the stressing condition leading to cell survival or triggering cell death in case of irreversible damages [39,40]. While dox and Caelyx® did not affect the expression of UPR-related gens in K7M2 cells, Sdox, Lsdox and HA-Lsdox up-regulated UPR-sensors and effectors (**Fig. 4a**). In parallel, they up-regulated most UPR-downstream mediators of cell death and down-regulated most UPR-downstream mediators of cell survival (**Fig. 4b**).

We previously demonstrated that in chemoresistant cells increases the activation of the ER stress-sensitive pro-apoptotic isoform of C/EBP-β (i.e. C/EBP-β LIP) and downstream effectors CHOP and TRB3 [41,42] up-regulate PUMA, that in turns activates caspases 12/7/3 [16,43,44]. Consistently, Sdox, Lsdox and HA-Lsdox activated the C/EBP-β LIP/CHOP/TRB3 axis, increased the expression of PUMA and the cleavage of caspases 12/7/3, while dox and Caelyx® were ineffective in Pgp-positive K7M2 cells (**Fig 4c**). Again, the most effective formulation was HA-Lsdox, as a consequence of the maximal drug delivery within ER achieved by this formulation.

Chemoresistant cells are usually refractory to commonly ER stressing agents including chemotherapeutic drugs, but genetic or pharmacologic tools able to re-activate the C/EBP-β LIP/CHOP re-instates a strong sensitivity to ER stress-mediated cell death [41,42,45]. To prove that the C/EBP-β LIP target CHOP was critical in inducing the ER-dependent apoptosis elicited by H_2S-releasing dox, we transiently silenced this protein in K7M2 cells. CHOP silencing abrogated the increase TRB3, PUMA, cleaved caspases 12, 7 and 3 in response to Sdox and HA-Lsdox (**Fig. 4d**), suggesting that CHOP was the *deus ex-machina* of ER-dependent apoptosis in osteosarcoma, as it occurred in other tumors [43-46].

3.5. Hyaluronated liposomes containing H_2S-releasing doxorubicin down-regulate Pgp via sulphydration and ubiquitination

An intriguing effect observed in HA-Lsdox-treated tumors was the strong decrease in Pgp expression (**Fig. 2c**).

While dox and Caelyx® did not modify the sulfhydration (**Fig. 5a**) nor the catalytic activity (**Fig. 5b**) of the Pgp isolated from ER, Sdox, Lsdox and HA-Lsdox progressively increased Pgp sulfhydration and decreased its ATPase activity (**Fig. 5a-b**). The reduction of Pgp activity was paralleled by a progressive increase in Pgp ubiquitination (**Fig. 5c-d**), maximally induced by HA-Lsdox. As for other ER-associated proteins, the release of H_2S was responsible for all these processes, since hydroxycobalamin prevented the Pgp sulphydration, decreased catalytic activity and ubiquitination induced by H_2S-releasing agents (**Fig. 5a-d**). Sulfhydration can activate or inhibit the target proteins [47,48]. During the folding, Pgp forms disulfide bonds that are critical to maintain a proper tertiary structure, stability and catalytic activity [48,49]. Our findings suggest that sulphydration destabilized Pgp and promoted its degradation, explaining the greater intracellular retention of Sdox formulations and the lower Vmax of Sdox efflux observed of Pgp-overexpressing osteosarcoma cells [15], suggestive of a decreased amount of Pgp.

3.6 Conclusion

In summary, we propose an effective and safe formulation against Pgp-overexpressing/dox-resistant osteosarcoma. The greater efficacy of HA-Lsdox relies on increased intracellular uptake, decreased efflux because of Pgp degradation, increased cell death triggered by ER stress. All these features were coupled with low cardiotoxic and systemic toxicity profile. For these reasons, HA-Lsdox is an innovative tool noteworthy to be tested in clinical settings, in particular in the osteosarcoma patients with high levels of Pgp and resistance to doxorubicin, where the rate of failure of the first-line chemotherapy is high.

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Figure 1. Hyaluronated liposomes containing H_2S-releasing doxorubicin effectively induce cell death in doxorubicin-resistant osteosarcoma cells

Human doxorubicin-sensitive U-2OS, their resistant subline U-2OS/DX580 and murine doxorubicin-resistant K7M2 cells were incubated 6 h (panel **c**), 2 h (panels **d-e**) or 96 h (panels **f**) in fresh medium (ctrl), in medium containing 5 μM doxorubicin (dox), liposomal doxorubicin Caelyx®, H_2S-releasing doxorubicin (Sdox), liposomal Sdox (Lsdox) or hyaluronated liposomal Sdox (HA-Lsdox). **a**. Expression of ABCB1/Pgp in untreated cells 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.** Surface CD44 in untreated cells, measured as per flow cytometry in duplicates. Blank: cells incubated with not-immune isotypic antibody. Histograms are representative of 1 out of 5 experiments. The percentage of CD44-positive cells as means ± SD is: 46±11% (U-2OS cells); 76±8% (U-2OS/DX580 cells); 69±7% (K7M2 cells). *p<0.05: U-2OS/DX580 and K7M2 cells vs. U-2OS cells. **c.** Intracellular doxorubicin accumulation, measured by a fluorimetric assay in duplicates. Data are means ± SD (n=4 independent experiments). °p<0.001: DX-cells vs. parental U-2OS cells; #p<0.001: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §p<0.002: HA-Lsdox vs Sdox; ◊p<0.001: HA-Lsdox vs Lsdox. **d.** Extracellular release of LDH measured spectrophotometrically in triplicates. Data are means ± SD (n=4 independent experiments). *p<0.01: treated vs. respective untreated (ctrl) cells; °p<0.001: DX-cells vs. parental U-2OS cells; #p<0.001: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §p<0.001: HA-Lsdox vs Sdox; ◊p<0.001: HA-Lsdox vs Lsdox. **e**. Activation of caspase 3 measured fluorimetrically in triplicates. Data are means ± SD (n=4 independent experiments). *p<0.01: treated vs. respective untreated (ctrl) cells; °p<0.001: DX-cells vs. parental U-2OS cells; #p<0.001: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §p<0.001: HA-Lsdox vs Sdox; ◊p<0.005: HA-Lsdox vs Lsdox. **f.** Percentage of viable cells, measured by a chemiluminescence-based assay in quadruplicates. Data are means ± SD (n=6 independent experiments). *p<0.01: treated vs. respective untreated (ctrl) cells; °p<0.001: DX-cells vs. parental

U-2OS cells; #$p<0.05$: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §$p<0.001$: HA-Lsdox vs. Sdox; ◊$p<0.005$: HA-Lsdox vs. Lsdox.

Figure 2. Hyaluronated liposomes containing H_2S-releasing doxorubicin reduce the growth of osteosarcoma refractory to doxorubicin and Caelyx®

K7M2 cells were subcutaneously implanted in 6-week old female Balb/C mice. When tumor reached the volume of 50 mm^3, animals were randomized in the following groups (n= 8/group) and treated as it follows at day 3, 9, 15 after randomization: 1) control group, treated with 200 µl sterile physiological solution, intravenously (i.v.); 2) dox group, treated with 200 µl sterile physiological solution containing 5 mg/kg dox, i.v.; 3) Caelyx® group, treated with 200 µl sterile solution of Caelyx®, equivalent to 5 mg/kg dox, i.v.; 4) Sdox group, treated with 200 µl sterile physiological solution containing 5 mg/kg Sdox, i.v.; 5) Lsdox group, treated with 200 µl sterile solution of Lsdox, equivalent to 5 mg/kg Sdox, i.v.; 6) HA-Lsdox group, treated with 200 µl sterile solution of HA-Lsdox, equivalent to 5 mg/kg Sdox, i.v. **a.** Tumor growth was monitored daily by caliper measurement. Data are presented as means ± SD. #$p<0.05$: Sdox/Lsdox/HA-Lsdox vs. ctrl/dox/Caelyx®; §$p<0.001$: HA-Lsdox vs. Sdox; ◊$p<0.005$: HA-Lsdox vs. Lsdox (days: 15-21). **b.** Photographs of representative tumors of each group. **c.** Immublot analysis of the indicated proteins in tumor homogenates. The β-tubulin expression was used as control of equal protein loading. The figure reports the results obtained in 3 animals/group of treatment.

Figure 3. Hyaluronated liposomes containing H_2S-releasing doxorubicin impair the ERAD/ERQC system of doxorubicin resistant osteosarcoma cells

a-b. Human doxorubicin-sensitive U-2OS cells and their resistant subline U-2OS/DX580 cells, murine doxorubicin-resistant osteosarcoma K7M2 cells were incubated 6 h with fresh medium (ctrl), medium containing 5 µM doxorubicin (dox), liposomal doxorubicin Caelyx®, H_2S-releasing doxorubicin (Sdox), liposomal Sdox (Lsdox) or hyaluronated liposomal Sdox (HA-Lsdox). After this incubation time, nuclear and microsomal fractions were extracted as reported in the Materials and Methods section. The intracellular doxorubicin accumulation was measured by a fluorimetric

assay in duplicates, in nuclear (panel **a**) and microsomal (panel **b**) extracts. Data are means ± SD (n=4 independent experiments). °p<0.02: DX-cells vs. parental U-2OS cells; #p<0.02: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §p<0.001: HA-Lsdox vs. Sdox; ◊p<0.001: HA-Lsdox v.s Lsdox. **c.** Hitmap of ER-associated degradation/endoplasmic reticulum quality control (ERAD/ERQC)-related genes in K7M2 cells, incubated as reported in **a** for 24 h. The figure reports genes up-or down-regulated at least two-fold compared to untreated cells, where the expression of each gene in untreated cells was considered 1 (not shown in the figure; n=6 independent experiments). **d.** Fluorimetric analysis of sulfhydrated microsomal proteins, performed in triplicates. When indicated, 100 µM of hydroxyl-cobalamin (Cbl), a H_2S scavenger, was co-incubated. Data are means ± SD (n=5 independent experiments). *p<0.01: treated vs. respective untreated (ctrl) cells; °p<0.001: Cbl-treated cells vs. corresponding Cbl-untreated cells; #p<0.001: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §p<0.002: HA-Lsdox vs Sdox; ◊p<0.002: HA-Lsdox vs Lsdox. **e.** Ubiquitination of microsomal proteins, measured by a chemiluminescence-based assay in triplicates, in K7M2 cells incubated as reported in **d**. Data are means ± SD (n=5 independent experiments). *p<0.01: treated vs. respective untreated (ctrl) cells; °p<0.001: Cbl-treated cells vs. corresponding Cbl-untreated cells; #p<0.001: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; §p<0.05: HA-Lsdox vs. Sdox; ◊p<0.05: HA-Lsdox vs. Lsdox.

Figure 4. Hyaluronated liposomes containing H_2S-releasing doxorubicin triggers a ER stress-dependent apoptosis in doxorubicin-resistant osteosarcoma cells

Murine doxorubicin-resistant osteosarcoma K7M2 cells were incubated 24 h with fresh medium (ctrl), medium containing 5 µM doxorubicin (dox), liposomal doxorubicin Caelyx®, H_2S-releasing doxorubicin (Sdox), liposomal Sdox (Lsdox) or hyaluronated liposomal Sdox (HA-Lsdox). **a.** Hitmap of unfolded protein response (UPR)-related genes. The figure reports genes up-or down-regulated at least two-fold compared to untreated cells, where the expression of each gene in untreated cells was considered 1 (not shown in the figure; n=6 independent experiments). **b.** Hitmap of genes related to ER-dependent cell death or survival. The figure reports genes up-or down-

regulated at least two-fold compared to untreated cells, where the expression of each gene in untreated cells was considered 1 (not shown in the figure; n=6 independent experiments). **c**. Cells treated as in **a** were lysed and probed with the indicated antibodies. The β-tubulin expression was used as control of equal protein loading. **d**. K7M2 cells were transfected with non-targeting scrambled siRNA pools (scr) or with siRNA pools targeting CHOP/GADD53 (siCHOP). 24 h after the transfection, the medium was changed and cells were grown for additional 24 h in fresh medium (ctrl) or in medium containing 5 μM dox, Sdox, HA-Lsdox. Whole cell lysates were probed with the indicated antibodies. The β-tubulin expression was used as control of equal protein loading. The figure is representative of 1 out of 4 experiments.

Figure 5. Hyaluronated liposomes containing H_2S-releasing doxorubicin reduce Pgp in doxorubicin-resistant osteosarcoma cells by increasing the protein sulphydration and ubiquitination

Murine doxorubicin-resistant osteosarcoma K7M2 cells were incubated 24 h with fresh medium (ctrl), medium containing 5 μM doxorubicin (dox), liposomal doxorubicin Caelyx®, H_2S-releasing doxorubicin (Sdox), liposomal Sdox (Lsdox) or hyaluronated liposomal Sdox (HA-Lsdox). When indicated, 100 μM of hydroxyl-cobalamin (Cbl), a H_2S scavenger, was co-incubated. **a.** Pgp was isolated by immunoprecipitation from microsomal extracts; the amount of sulfhydrated Pgp was measured fluorimetrically in triplicates. Data are means ± SD (n=5 independent experiments). $^*p<0.01$: treated vs. respective untreated (ctrl) cells; $^\circ p<0.001$: Cbl-treated cells vs. corresponding Cbl-untreated cells; $^\# p<0.001$: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; $^\S p<0.001$: HA-Lsdox vs. Sdox; $^\Diamond p<0.001$: HA-Lsdox vs. Lsdox. **b.** Pgp ATPase activity was measured spectrophotometrically in triplicates, on Pgp isolated by immunoprecipitation from microsomal extracts. Data are means ± SD (n=5 independent experiments). $^*p<0.01$: treated vs. respective untreated (ctrl) cells; $^\circ p<0.001$: Cbl-treated cells vs. corresponding Cbl-untreated cells; $^\# p<0.001$: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; $^\S p<0.002$: HA-Lsdox vs Sdox; $^\Diamond p<0.002$: HA-Lsdox vs. Lsdox. **b.** Ubiquitinated Pgp, isolated by immunoprecipitation from microsomal extracts, measured

by a chemiluminescense-based assay in triplicates. Data are means ± SD (n=5 independent experiments). *$p<0.01$: treated vs. respective untreated (ctrl) cells; °$p<0.001$: Cbl-treated cells vs. corresponding Cbl-untreated cells; $^{\#}p<0.001$: Sdox/Lsdox/HA-Lsdox vs. dox/Caelyx®; $^{\S}p<0.002$: HA-Lsdox vs. Sdox; $^{\Diamond}p<0.002$: HA-Lsdox vs. Lsdox. **c.** Microsomal proteins were immunoprecipitated (IP) with an anti-Pgp antibody, then immunoblotted (IB) with an anti-mono/poly-ubiquitin (UQ) antibody. An aliquot of the samples before immunoprecipitation was probed with an anti-Pgp antibody or with an anti-calreticulin antibody, used as control of equal protein loading. The figure is representative of 1 out of 4 experiments. no Ab: untreated cell lysate immunoprecipitated in the absence of antibody, to check the specificity of the procedure. MW: molecular weight. The figure is representative of 1 out of 4 experiments.

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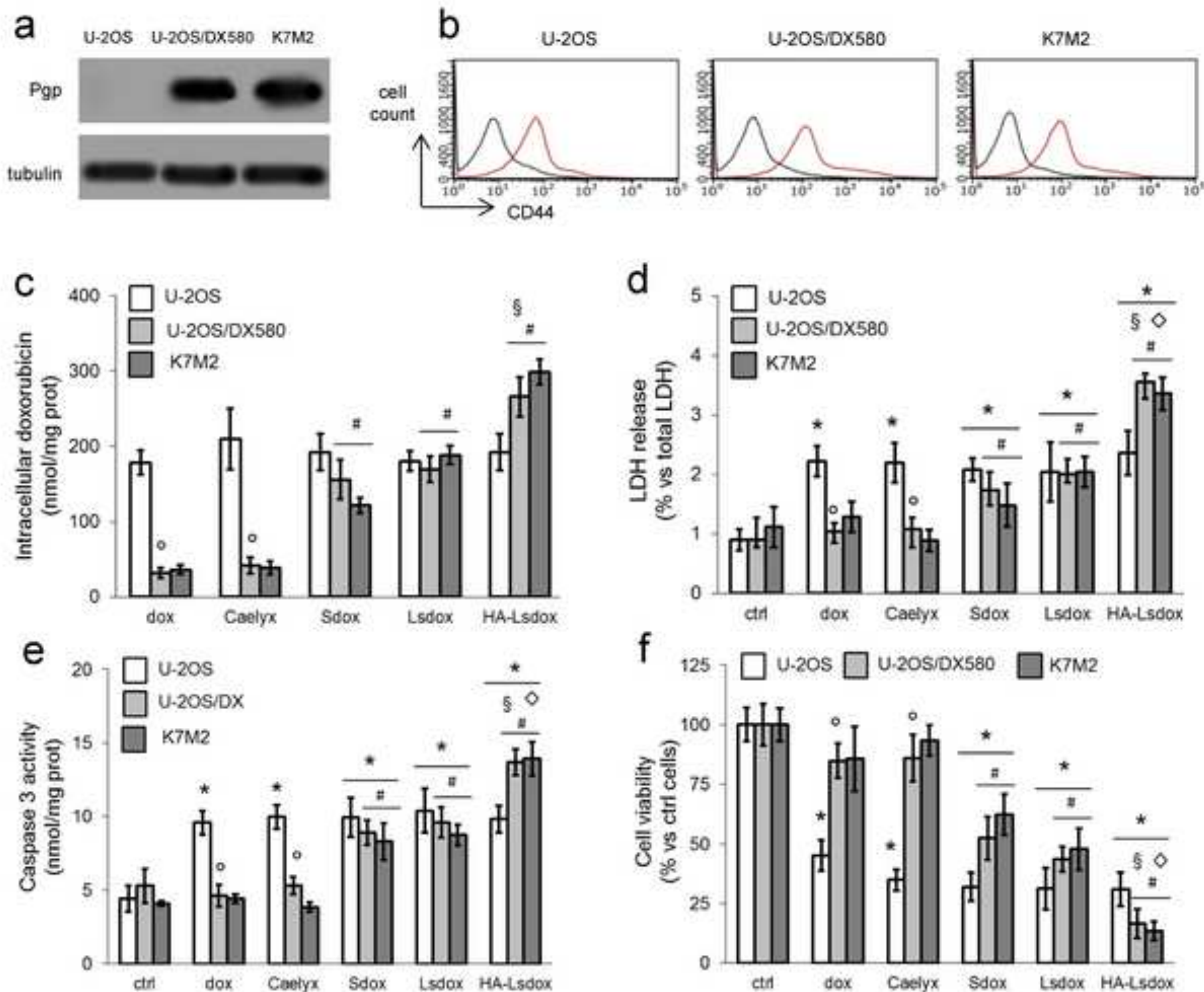

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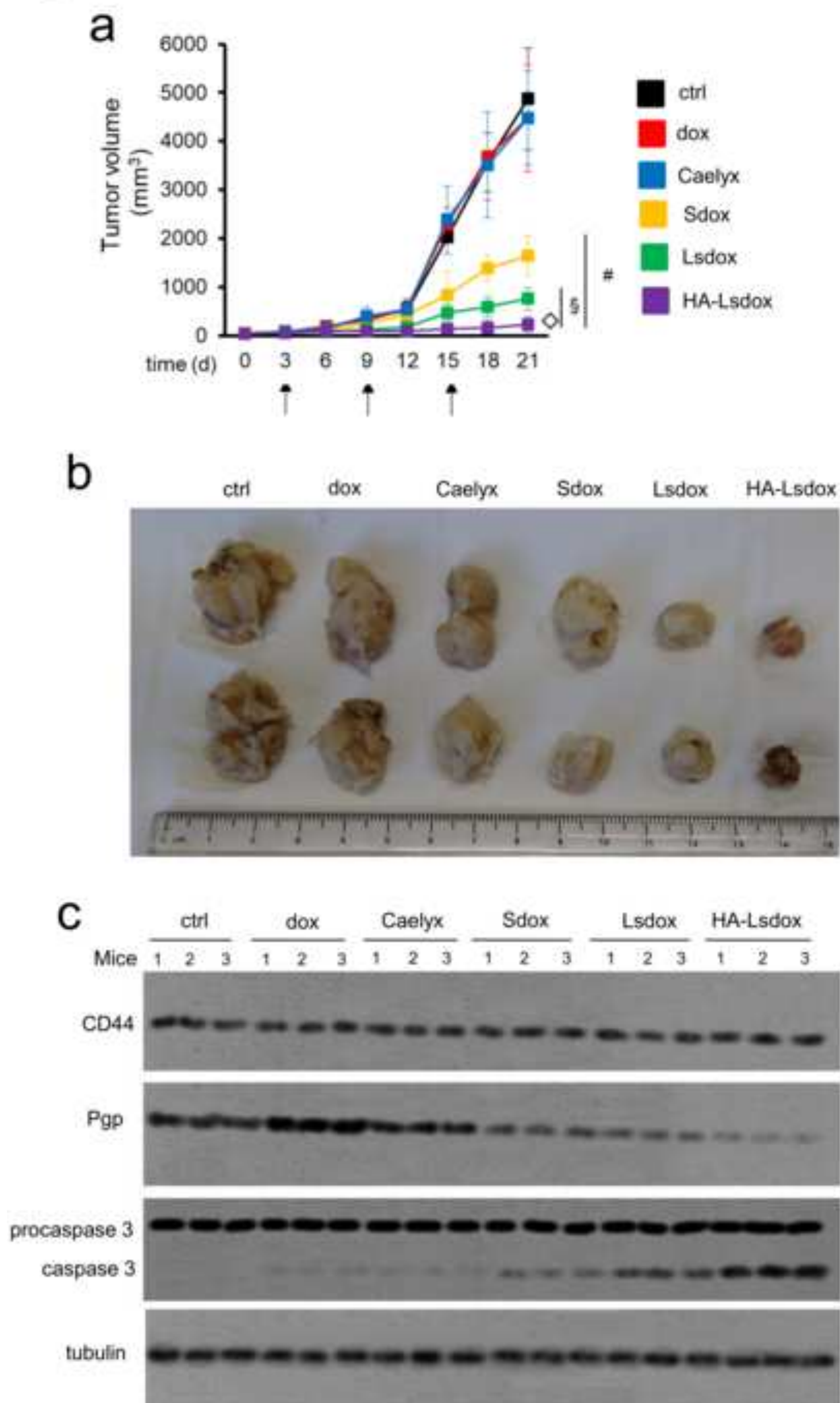

Figure 3
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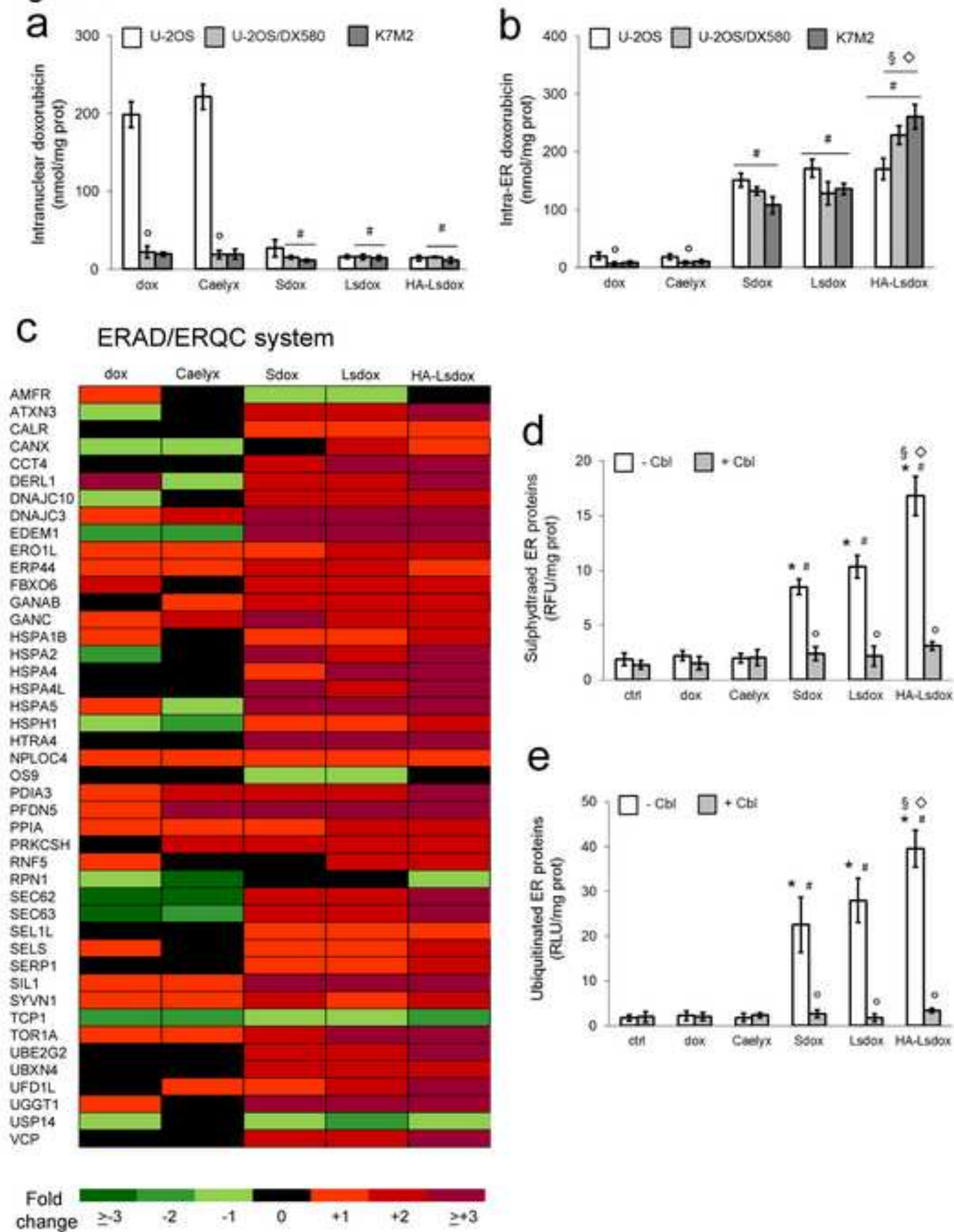

Figure 4

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Figure 4

a UPR

dox Caelyx Sdox Lsdox HA-Lsdox

ATF4
ATF6
ATF6B
CEBPB
CREB3L3
DDIT3
EIF2A
EIF2AK3
ERN1
GADD53
HERPUD1
NUCB1
TRIB3
XBP1

Fold change ≥-3 -2 -1 v 0 +1 +2 ≥+3

b Cell death

dox Caelyx Sdox Lsdox HA-Lsdox

BAX
BEX2
CREB3
ERN2
ERO1LB
HTRA2
INHBE
MANF
UHRF1

Cell survival

dox Caelyx Sdox Lsdox HA-Lsdox

DNAJB9
GINS2
MAPK8
MAPK9
MCM4
PCNA
PPP1R15A
RRM2
TYMS

Fold change ≥-3 -2 -1 v 0 +1 +2 ≥+3

c

ctrl dox Caelyx Sdox Lsdox HA-Lsdox

CEBP/β LAP
CEBP/β LIP
CHOP
TRB3
PUMA
procaspase 12
caspase 12
procaspase 7
caspase 7
procaspase 3
caspase 3
tubulin

d

scr: ctrl dox Sdox HA-LsDox
siCHOP: ctrl dox Sdox HA-Lsdox

CHOP
TRB3
PUMA
procaspase 12
caspase 12
procaspase 7
caspase 7
procaspase 3
caspase 3
tubulin

Figure 5
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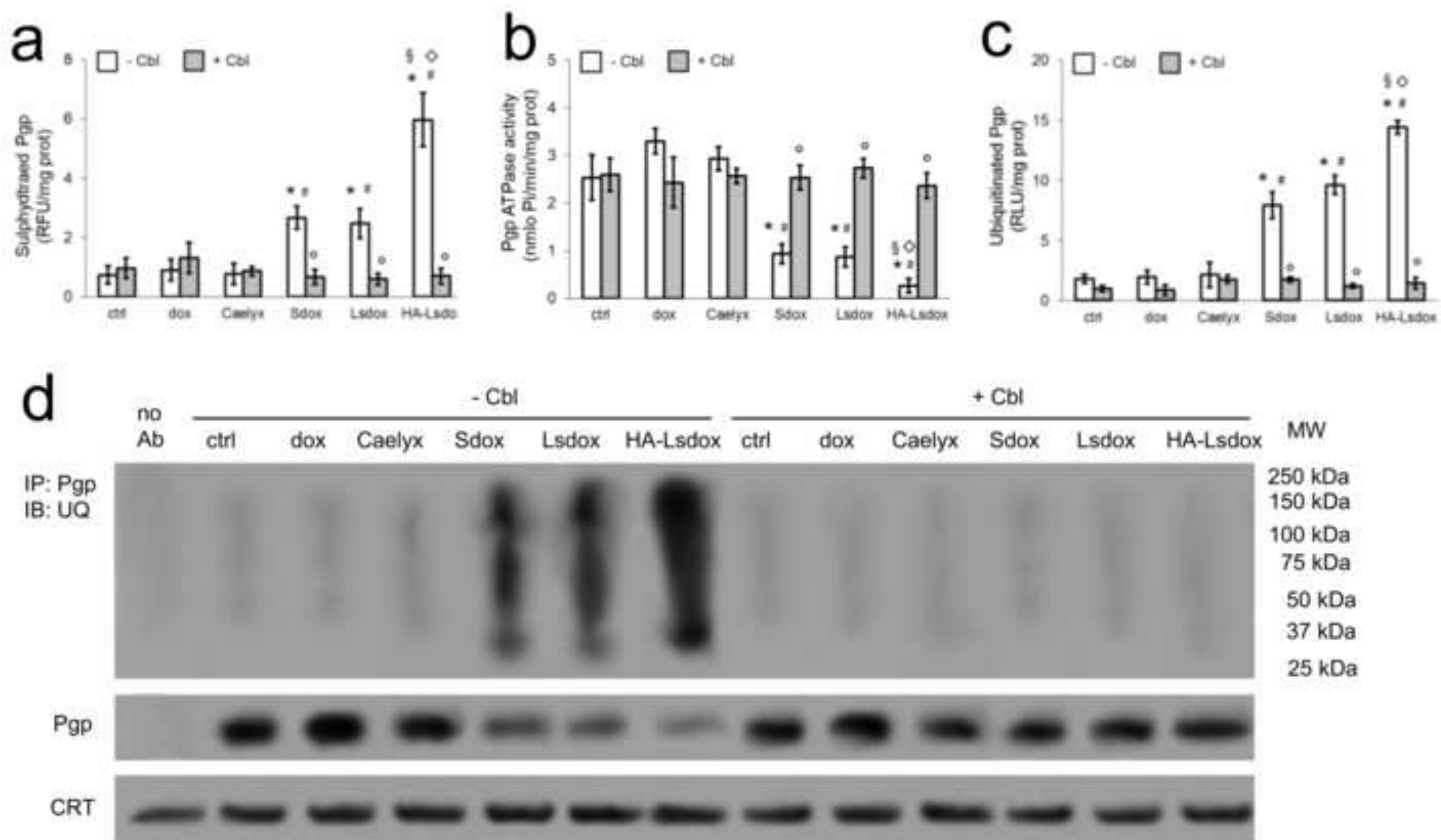

Table 1. Characteristics of liposomes containing Sdox (means ± SD; n=3).

Formulation	Mean particle size (nm ± SD)	Polydispersity Index	Zeta potential (mV ± SD)	Entrapment efficiency (%)*
Lsdox	190 ± 3	0.112	-7.51 ± 0.8	92.8 ± 3.2
HA-Lsdox	204 ± 2	0.121	-15.8 ± 1.3	91.3 ± 3.1

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

Supplementary File

Click here to download Supplementary File: Cancer Letter_Supplementary Materials.docx

*Conflicts of Interest Statement
Click here to download Conflicts of Interest Statement: Conflict of interest.docx

Conflict of interest

Declarations of interest: none.