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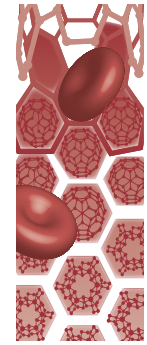

# Exosomes increase the therapeutic index of doxorubicin in breast and ovarian cancer mouse models

**Aim:** To demonstrate that exosomes (exo) could increase the therapeutic index of doxorubicin (DOX). **Materials & methods:** Exosomes were characterized by nanoparticle tracking analysis and western blot. Tissue toxicity was evaluated by histopathological analysis and drug efficacy by measuring tumor volume. DOX biodistribution was analyzed by MS. **Results:** Exosomal doxorubicin (exoDOX) avoids heart toxicity by partially limiting the crossing of DOX through the myocardial endothelial cells. For this reason, mice can be treated with higher concentration of exoDOX thus increasing the efficacy of DOX as demonstrated in breast and ovarian mouse tumors. **Conclusion:** ExoDOX is safer and more effective than free DOX. Importantly, the first spontaneous transformed syngeneic model of high-grade serous ovarian cancer was utilized for providing a new therapeutic opportunity.

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**Keywords:** breast and ovarian cancers • doxorubicin • drug delivery • exosomes • therapeutic index

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Cancer patients need better therapeutic opportunities in terms of efficacy and compliance. Most of the chemotherapeutic drugs have a narrow therapeutic window, a range of doses that produces therapeutic response without causing any significant adverse effect in patients [1]. Many variables can influence the therapeutic window and among them, drug formulation, off-target effects, biochemical and genetic characteristics of the patients were thoroughly studied in the last decade [2]. Personalized medicine, a tailored approach to cure individual patients, is the optimal choice to overcome these limitations [3,4]. From one side, many laboratories and pharmaceutical companies are currently focusing on the genomic and genetic characteristics of the tumors [5,6]. On the other side, nanomaterials hold great promises for cancer patients but also face major challenges to be translated into clinic [7–11]. Organic materials such as liposomes have been a breakthrough in the field of drug delivery but also offer many limits such as reproducibility, organ toxicity and/or immune response, which have limited their application [12–14].

Nature offers many opportunities for new drug vehicles. Normal and cancerous cells communicate with each other and their environment both locally and at great distance. Among the mechanism of communication, extracellular vesicles have been recognized as an emerging new class of vehicles [15,16]. Exosomes are a subclass of extracellular vesicles that represent an extraordinary material rich of information for diagnostic applications and therapeutic opportunities [17]. It has been recently demonstrated that tumoral exosomes from pancreatic cancer are enriched in a cell surface proteoglycan, glypican-1, which distinguishes healthy subjects and patients with benign pancreatic tumors from patients with early- and late-

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stage pancreatic cancer, and could therefore be used as a potential noninvasive diagnostic and screening tool [18]. Moreover, besides being a diagnostic tool, exosomes are ideal drug delivery agents. The size ranging from 30 to 200 nm has been demonstrated to be optimal for long circulating time thereby avoiding fast clearance [19]. They can freely circulate and distribute into biological fluids such as blood, urine, ascites, saliva and cerebrospinal fluid. The membrane composition is similar to that of the cell of origin, which contains a specific set of lipids and proteins with a potential quasi-infinite number of different vesicles with unique characteristics. The membrane composition shares optimal fusogenic properties with cell membranes and in the same instances may exhibit a specific cell tropism [20,21]. Exosomes derived from patients can avoid immune surveillance better than *in vitro* formulated pegylated liposomes [21–23]. Cells utilize exosomes to communicate with the environment and transfer DNA, RNA, proteins, lipids and metabolites [17,24–26]. For these reasons, exosomes have been utilized to deliver nucleic acids to the brain, small molecules or proteins [27–30].

Based on previous observations [28], we sought to determine whether exosomes could increase the therapeutic index of DOX. By utilizing a breast and a new syngeneic mouse model of ovarian cancer [31], it was demonstrated that exosomes represent an effective drug delivery system to be used in cancer treatment.

## Experimental section

### Reagents

MDA-MB-231 (human breast cancer) and STOSE (mouse ovarian cancer) cell lines were grown as indicated by the supplier. MDA-MB-231 CD63-GFP and STOSE CD63-GFP cell lines were prepared by transduction of MDA-MB-231 and STOSE cell lines with lentivirus containing CD63-GFP plasmid purchased from System Biosciences (CA, USA).

Antibodies: α-tubulin (TUBA1A; T9026, 1:10,000) from Sigma-Aldrich (MO, USA); Flotillin1 (FLOT1; ab41927, 1:1000) from Abcam (Cambridge, UK); Lamp1 (9091S, 1:1000) from Cell Signaling (MA, USA). Secondary antibodies were from Thermo Fisher Scientific (MA, USA): antirabbit (31464, 1:10,000) and antimouse (31432,1:10,000).

Nude and FVB/N mice were purchased from Harlan Laboratories (Udine, Italy). The experimental procedures were approved by the Italian Ministry of Health no. 788/2015-PR and performed in accordance with the institutional guidelines. We utilized at least three female mice of 8 weeks of age per data point. Data are reported as mean and standard error of the mean.

### Exosomes loading & characterization

Exosomes from MDA-MB-231, STOSE, MDA-MB-231 CD63-GFP and STOSE CD63-GFP cell lines were prepared from exosome-depleted medium conditioned for 48 h and purified with AB CELL CULTURE-Nanovesicles solution according to the instructions (AB ANALITICA, Padova, Italy). Exosomes quantification was done by the Bradford method. A total of 200 µg of exosomes were mixed with 200 µg of DOX in electroporation buffer (1.15 mM potassium phosphate, 25 mM potassium chloride, 21% Optiprep) and electroporated at 150 V, 0.125 × 1000 µF under max capacitance in a 0.4-cm cuvette. Exosomal doxorubicin (exoDOX) were collected by centrifugation and washed three-times with PBS 1X. DOX concentration in exosomes was quantified by measuring the absorbance at 490 nm using Tecan F200 instrument (Tecan, Männedorf, Switzerland). The loading efficiency of DOX in exosomes was 2.56 ± 0.57% (encapsulated/total). To load 1 µg of DOX, 22.5 ± 6.64 µg of exosomes were utilized. The release of DOX and exoDOX were evaluated with a dialysis membrane of 20.000 MWCO that had been dipped into 50% fetal bovine serum at pH 7.4.

Exosomes were characterized by Scanning Electron Microscopy. They were dehydrated in a graded 30–100% ethanol series, dried in a $CO_2$ apparatus at a critical point (Bal-Tec; EM Technology and Application, Liechtenstein), sputter coated with gold in an Edwards S150A apparatus (Edwards High Vacuum, UK), and examined with a Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd, UK).

Exosomes size was determined by nanoparticle tracking analysis (NTA) with a NanoSight LM10 instrument (Malvern, UK) in PBS 1× buffer.

### Fluorescence imaging of exosomes from MDA-MB-231 CD63-GFP & STOSE CD63-GFP cells

To prepare MDA-MB-231 CD63-GFP and STOSE CD63-GFP cells, $5 \times 10^5$ cells were plated in a 10-cm plate (Becton Dickinson, NJ, USA), incubated for 24 h to allow cells attachment. Cells were transduced with 1 ml of lentivirus containing CD63-GFP fusion protein (approximately 1 virus/cell) and hexadimethrine bromide [32,33]. Two days later, cells were analyzed by fluorescence microscopy using a filter set with Ex 490/Em 520 nm wavelengths. MDA-MB-231 CD63-GFP and STOSE CD63-GFP cells were incubated in exosome-free medium for 48 h; the medium was then collected and exosomes were extracted using AB CELL CULTURE-Nanovesicles solution according to the instructions (AB ANALITICA, Padova, Italy). The exosomes were diluted 1:100 in PBS 1× and a drop was laid on cover slip and analyzed by fluorescence microscopy.

### Western blot

The cell pellets and exosomes (from 1.5 $10^7$ cells) were resuspended into radioimmunoprecipitation assay buffer supplemented with a protease inhibitor mixture (Complete-EDTA, Roche, Switzerland) for protein extraction and 50 µg of proteins were run in 8% denaturating polyacrylamide gel. After electrophoresis, the proteins were transferred on nitrocellulose membrane (Whatman International Ltd, UK). The membranes were blocked with 5% (w/v) skim milk in Tris-buffered saline Tween-20 solution (TBS-T) and incubated overnight with primary antibodies (Lamp1, FLOT1 and TUBA1A). After washing, the membranes were incubated for 1 h with secondary antibodies in 5% milk TBS-T at RT. The membranes were developed with ECL solution (Euroclone, Italy) and visualized with ChemiDoc Imager instrument (Bio-Rad Laboratories, CA, USA) [34].

### Human cardiac microvascular endothelial cells Transwell assay

To study the ability of exoDOX to cross endothelial cell barrier, a Transwell system was carried out. Human cardiac microvascular endothelial cells (PromoCell, Germany) were plated at the density of $10^5$ cells on cell culture insert with pore size of 8 µm (Becton Dickinson, NJ, USA) pretreated with 5 µg/$cm^2$ of fibronectin (Sigma-Aldrich, MO, USA). The next day, two concentrations of 10 µg/ml and 50 µg/ml of both exoDOX and DOX were added on the top of cell-culture insert in phenol red free medium and the concentration of DOX was read from the bottom up by measuring the absorbance at 490 nm at different time points (0, 0.5, 2, 4, 7, 16 and 28 h).

### The half maximal inhibitory concentration & cell viability assay

In order to evaluate the half maximal inhibitory concentration ($IC_{50}$) of DOX, cells were plated with a density of $10^3$ cells/well in a 96-wells plate (Becton Dickinson, NJ, USA) and incubated for 24 h to allow the attachment of cells. The next day, cells were treated with DOX starting with a concentration of 1 µg/ml followed by five 1:10 serial dilutions. After 96 h, the cell viability was evaluated by CellTiter-Glo® Luminescence assay (Promega, WI, USA) with the Infinite 200 PRO instrument (Tecan) and $IC_{50}$ was calculated using the GraphPad program (Prism, CA, USA).

Cytotoxicity of free DOX and exoDOX were tested in STOSE cells according to $IC_{50}$. Cells were seeded in 96-wells plates (Becton Dickinson, NJ, USA) at a density of $10^3$ cells/well and incubated for 24 h to allow the attachment of cells. The cells were incubated with DOX, exosomes and exoDOX at the same drug concentrations for 96 h. The cytotoxicity was evaluated by CellTiter-Glo Luminescence assay (Promega, WI, USA) with the Infinite 200 PRO instrument (Tecan).

### Maximum tolerated dose

Nude mice were treated intraperitoneally (ip.) five-times, bi-weekly, with 1.5, 3, 5 and 7 mg/kg of DOX, Doxil and exoDOX (exosomes isolated from MDA-MB-231 cells), respectively. Another experiment was done to evaluate the maximum tolerated dose in FVB/N mice by comparing exoDOX (exosomes isolated from STOSE cells) and free DOX. The mice were treated intraperitoneally bi-weekly, with 3 and 6 mg/kg of DOX and exoDOX, respectively (five injections). Mice weight was measured for 20 days. A loss of more than 20% of body weight, lacks of grooming and hunched posture were considered as an end point. At the end of the experiment, organs from nude and FVB/N mice were isolated for histopathological analysis.

### Histopathology

Organs of mice were collected and fixed in phosphate-buffered 10% formalin, embedded in paraffin, sectioned at a thickness of 3 µm and stained with hematoxylin and eosin (H&E). The tissues were analyzed with light microscopy using different magnifications. Morphological details were analyzed at 40× objective.

### Mouse xenograft

The 3 × $10^6$ MDA-MB-231 cells were mixed with 30% of Matrigel (BD Bioscience, CA, USA) and implanted subcutaneously into 8-week-old female nude mice. 5 × $10^6$ STOSE cells were mixed with 30% of Matrigel (BD Bioscience) and implanted subcutaneously into the flanks of 8-week-old female FVB/N mice. when tumors reached a measurable size (>50 $mm^3$), mice were treated intraperitoneally with DOX, Doxil and exoDOX two-times per week for five treatments. Tumor volumes were measured with a caliper and calculated using the formula: (length × $width^2$)/2.

### Biodistribution

The tumors of mice were washed with 10 ml of cold PBS/heparin before collection, diluted in 500 µl of PBS/BSA 4% and homogenized with Qiagen Tissue Ruptor for 20 s at power 4 in ice. Samples were then stored at -80°C. The concentrations of DOX were measured by liquid chromatography tandem MS (LC-MS/MS). The proteins were precipitated with two volumes of cold acetonitrile containing 20 ng/ml daunorubicin as the internal standard. After vortexing and spinning at 13,000 rpm for 15 min at 4°C, the

cleared supernatant was diluted with two volumes of 0.2% formic acid and 10 µl were injected on LC-MS/MS system. Chromatographic separation was performed on Accucore-150 30 × 2.1 mm 2.6 µm C18 column (Thermo Scientific, MA USA), equilibrated with a 0.7 ml/min of 0.2% formic/acetonitrile (95:5) and maintained at 50°C. An elution gradient B from 5 to 80% of acetonitrile over 5 min was applied and 3 min of equilibration A 4000 QTRAP MS/MS system equipped with Turbo ESI source (AB Sciex, MA, USA) was applied in positive-ion mode. The transitions of DOX and daunorubicin were monitored in multireaction monitoring mode at m/z 544.1→397.2 and 528.2→321.1, respectively. The spray voltage was set at 5000 V and the source temperature at 400°C. The curtain gas, nebulizer gas (gas1) and auxiliary gas (gas 2) were set at 20, 50 and 50 arbitrary units, respectively. The declustering potential and collision energy voltages for both DOX and daunorubicin were set at 45 V and 16 V, respectively.

### Statistical analysis

The statistical significance was determined using the *t*-test. A p-value less than 0.05 was considered significant for all comparisons made.

## Results & discussion

### ExoDOX has a fewer aptitude to cross myocardial endothelial cells than free DOX

In a previously published paper, we demonstrated that exosomes isolated from different cancer cell lines could be utilized to reduce the toxicity associated to DOX [28]. Among the side effects, cardiotoxicity is a major problem caused by anthracyclines [1]. Since less exoDOX was found in the heart, it was speculated that myocardial vessels with tight junctions and the well-developed lymphatic system reduced the accumulation of exosomes [35]. We, therefore, isolated exosomes from MDA-MB-231 breast cancer cells, analyzed by NTA (Supplementary Figure 1), Scanning Electron Microscopy (Supplementary Figure 3) and western blot using Lamp1, FLOT1 and TUBA1A markers (Supplementary Figure 4) and loaded with DOX as previously described [28]. Endothelial myocardial cells were plated into Transwell membrane inserts (pore size 8 µm) coated with fibronectin (Figure 1A). DOX and exoDOX (10 and 50 µg) were added into the chamber insert and the absorbance was read from the bottom up. A time point analysis showed that at both concentrations, exoDOX has a lower ability to cross myocardial endothelia cells than free DOX (Figure 1B & C). Although

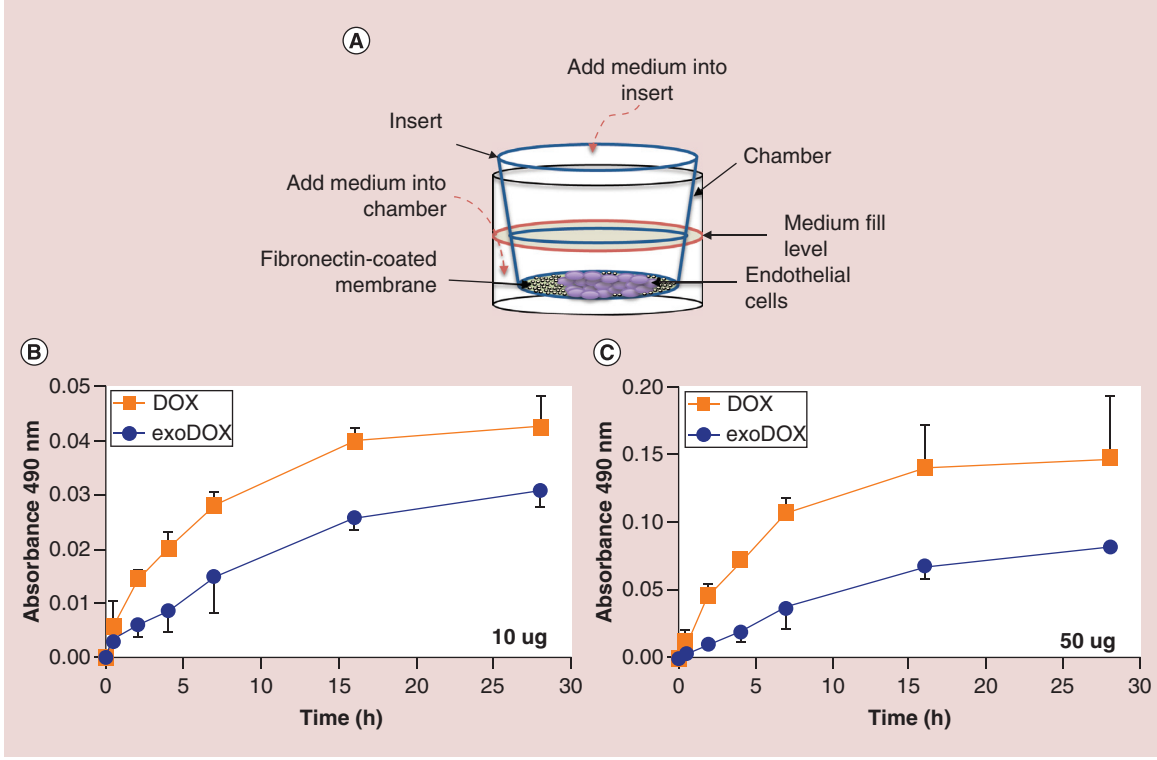


**Figure 1. Myocardial endothelial cells transwell assay. (A)** Schematic picture of Transwell describing the ability of DOX and exoDOX to cross a reconstructed myocardial endothelial monolayer. Absorbance of **(B)** 10 µg and **(C)** 50 µg of DOX and exoDOX in the lower chamber at seven time points (0, 0.5, 2, 4, 7, 16 and 28 h). Experiments were run in duplicates. Mean and standard deviation are reported.
DOX: Doxorubicin.

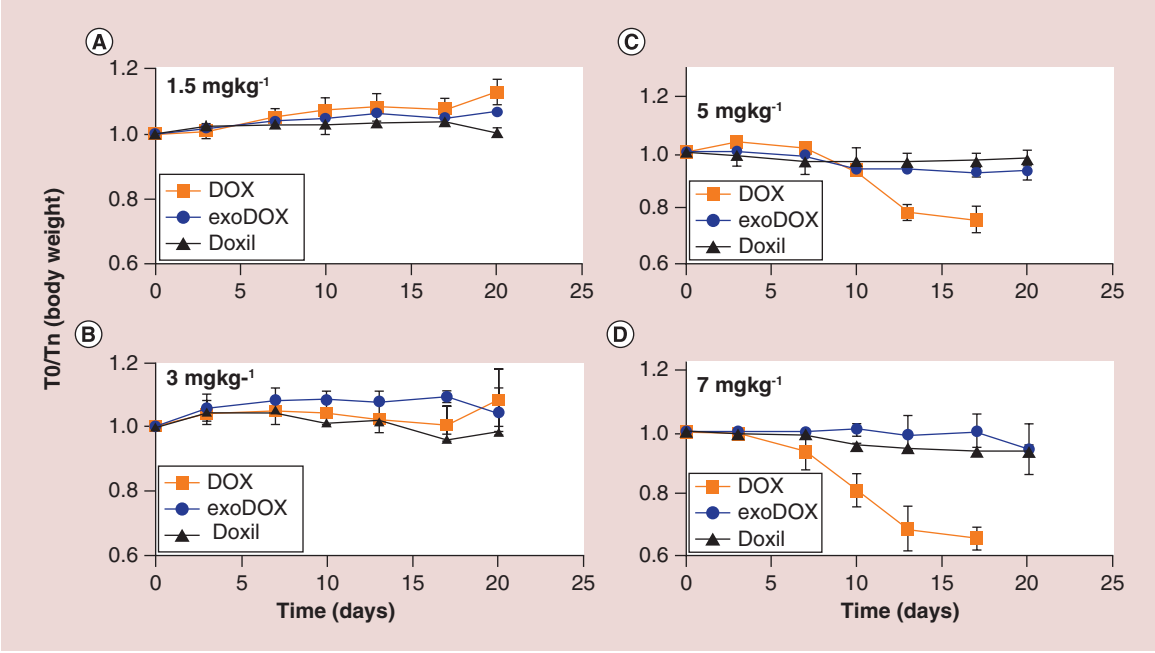


**Figure 2. Maximum tolerated dose experiment in nude mice utilizing four doses of DOX, Doxil and exoDOX.**
**(A)** 1.5 mg/kg, **(B)** 3 mg/kg, **(C)** 5 mg/kg and **(D)** 7 mg/kg; body weight was monitored at the indicated time points for 20 days.
DOX: Doxorubicin; MTD: Maximum tolerated dose.

the experiment was performed under static conditions, it was demonstrated the aptitude of exosomes to avoid extravasation in normal heart tissue.

## Exosomes increase tolerability of DOX in mouse

It is important to determine the stability of exoDOX in serum before *in vivo* application. A time point analysis of exosomes loaded with DOX was performed in 50% serum utilizing a semipermeable membrane (Supplementary Figure 5). A two-phase kinetic of release was observed. In the first 2 h, the release of exoDOX is similar to free DOX. Later, the release of DOX from exosomes was slow and never reached 100% over a period of 24 h. This difference in release could be explained by the interaction of DOX with different biological content of exosomes such as DNA, RNA and membranes.

Subsequently, nude mice were treated with different concentrations of exoDOX, Doxil (liposomal DOX) or free DOX as indicated in Figure 2. The drug was injected intraperitoneally, two-times per week for a total of five injections. As objective scale, we measured the body weight of mice: up to 3 mg/kg, there was no difference between treatments and no adverse effects were observed (Figure 2A & B). At 5 and 7 mg/kg, the mice treated with DOX started to lose weight after 10 days and finally they were sacrificed. ExoDOX- and Doxil-treated mice were healthy at all tested concentrations (Figure 2C & D).

## Exosomes increase DOX efficacy in a mouse model of breast cancer

Following the experiments of toxicity, it was decided to treat the mice with a double concentration of exoDOX compared with free DOX. MDA-MB-231 cell lines were injected subcutaneously in nude mice and when the tumors reached an average size >50 $mm^3$, exoDOX (6 mg/kg), Doxil (6 mg/kg) and DOX (3 mg/kg) were injected intraperitoneally two-times per week for a total of five treatments. The tumors treated with exoDOX and Doxil clearly had not grown compared with the DOX treated mice (Figure 3A). At the end of the experiment, DOX in the tumor was quantified by MS. Figure 3B shows that the concentration of exoDOX in the tumor had doubled compared with DOX concentration ($p$-value < 0.05).

## Exosomes increase DOX efficacy in an immunocompetent mouse model of high-grade serous ovarian cancer

Ovarian cancer is a lethal disease. Scientists have worked for years to establish a representative mouse model. Recently, Dr. Barbara Vanderhyden's group has developed a spontaneously transformed mouse ovarian surface epithelial cell line (STOSE), which closely recapitulates the characteristics of human high-grade serous ovarian cancer (HGSOC) [31]. The exosomes from STOSE cell lines were collected as described for MDA-MB-231 cell lines and characterized by NTA

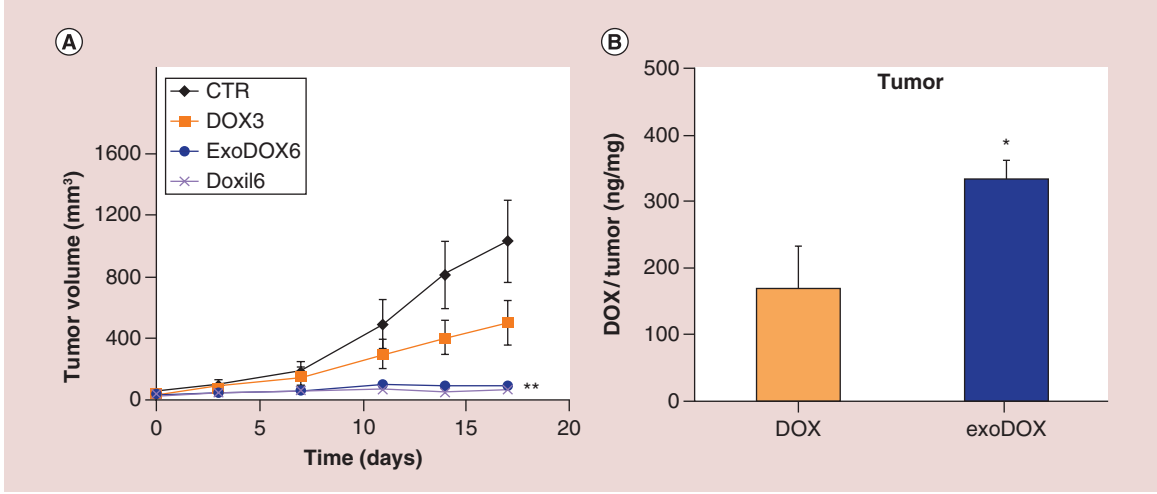


**Figure 3. ExoDOX efficacy on MDA-MB-231 tumor growth. (A)** Tumor volume in nude mice treated by ip. injection with (3 mg/kg) DOX (■), (6 mg/kg) exoDOX (•), (6 mg/kg) Doxil (×) and controls (♦). **(B)** In the tumors (3 h), exoDOX accumulated more compared with the DOX-treated mice. Y axis: concentration of DOX per gram of tissue. *$p < 0.05$.
DOX: Doxorubicin; ip.: Intraperitoneally.

analysis and western blot. The exosomes have a diameter of 101 nm and express the typical exosome marker FLOT1 (Supplementary Figures 2 & 3). To further confirm exosomes purification, cells were infected with CD63-GFP fusion protein (exosomal marker) and the isolated exosomes were analyzed under microscope. Fluorescence analysis of MDA-MB-231 and STOSE exosomes showed a dotted appearance as expected in vesicular isolation (Supplementary Figure 6).

Because STOSE cells closely recapitulate HGSOC, it was decided to validate the data obtained with MDA-MB-231 breast cancer cells. First, we calculated the $IC_{50}$ of DOX on both cell lines. $IC_{50}$ analysis of DOX demonstrated a similar sensitivity of STOSE and MDA-MB-231 cell lines. The average of $IC_{50}$ was 18.78 ± 1.25 and 13.85 ± 1.22 ng/ml for STOSE and MDA-MB-231 cell lines, respectively (Supplementary Figure 7). Treatment of STOSE cells with different concentrations of DOX and exoDOX showed the same effect on cell viability (Supplementary Figure 8) as previously demonstrated with MDA-MB-231 cell lines [28].

The tolerability of exoDOX was tested in isogenic FVB/N mice. The mice were treated with DOX and exoDOX at 3 and 6 mg/kg, two times per week for a total of five treatments (Figure 4A & B). Similarly to nude mice, at 6 mg/kg, the DOX-treated mice lost almost 20% of their body weight although the effect was less evident than in nude mice. Histopathological analyses demonstrated that the hearts of DOX-treated mice were normal at 3 mg/kg but showed vacuoles and moderate

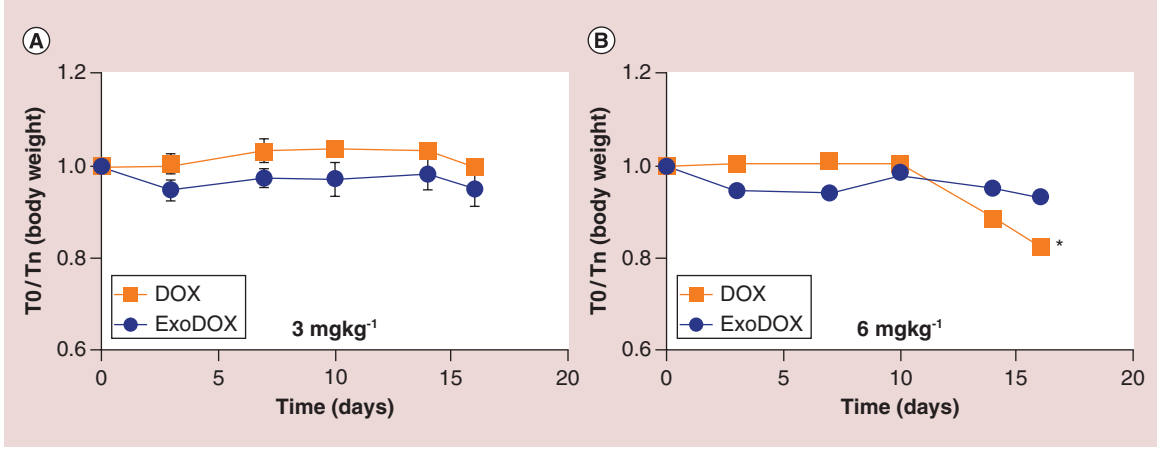


**Figure 4. MTD experiment in FVB/N mice using 2 doses of DOX and exoDOX, (A) 3 mg/kg, (B) 6 mg/kg. Body weight was monitored at the indicated time points for 16 days.**
DOX: Doxorubicin; MTD: Maximum tolerated dose.

myofibrils disorganization at 6 mg/kg (Figure 5A & B). In exoDOX-treated mice, the heart appears normal as control (Figure 5C & D). All other tissues were apparently normal (Supplementary Figure 9).

STOSE cell lines were inoculated subcutaneously in FVB/N mice to demonstrate the efficacy of exoDOX. After the tumor reached a size >50 $mm^3$, mice were treated with 3 mg/kg of DOX and 3 or 6 mg/kg of exoDOX (Figure 6A). At higher concentration, the exoDOX was more effective than free DOX, similar to MDA-MB-231 breast cancer model. Tumors analysis showed that at 6 mg/kg, exoDOX accumulated about two-times more than free DOX (Figure 6B; p-value < 0.05).

## Conclusion

In this paper, we demonstrate the utility of exosomes to deliver DOX in highly devastating female cancers. We carried out *in vitro and in vivo* experiments to provide conclusive evidences, which revealed that: similar to liposomes, myocardial endothelial cells limit exoDOX crossing, avoiding accumulation of drug in the heart. The kinetics of myocardial endothelial extravasation are slower in exoDOX compared with DOX; the maximum tolerated dose in immunodeficient and immunocompetent mice of exoDOX is higher than in free drug, thus limiting the cardiac toxicity without affecting other organs; exosomes increase the therapeutic index of DOX in breast and ovarian cancer mouse models (Figure 7).

## Future perspective

Breast cancer is one of the most common cancers. Breast and ovarian cancers account for more than 500,000 new cases every year [36–38]. Chemotherapeutic drugs are largely used as the standard therapy in both types of tumor with many side effects and DOX is not exempt. Among adverse effects, DOX cardiotoxicity limits its use, and has a poor prognosis and a frequent fatality [39]. The encapsulation of DOX in liposomes has improved the therapeutic index of this drug through a better circulation time and biodistribution. Nonetheless, the application of liposomes was modest: this is due to the low reproducibility during manufacturing, the difficulty to actively target tumor cells and their recognition

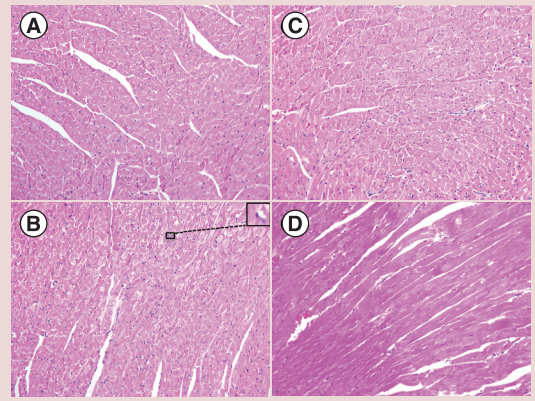


**Figure 5. ExoDOX-treated mice have normal cardiac tissue after maximum tolerated dose.** FVB/N mice were treated at **(A)** 3 mg/kg, **(B)** 6 mg/kg of DOX, **(C)** 6 mg/kg of exoDOX or **(D)** control. In **(B)** scattered cytoplasmic paranuclear vacuoles are zoomed in H&E staining. Original magnification, 40×.
DOX: Doxorubicin; MTD: Maximum tolerated dose.

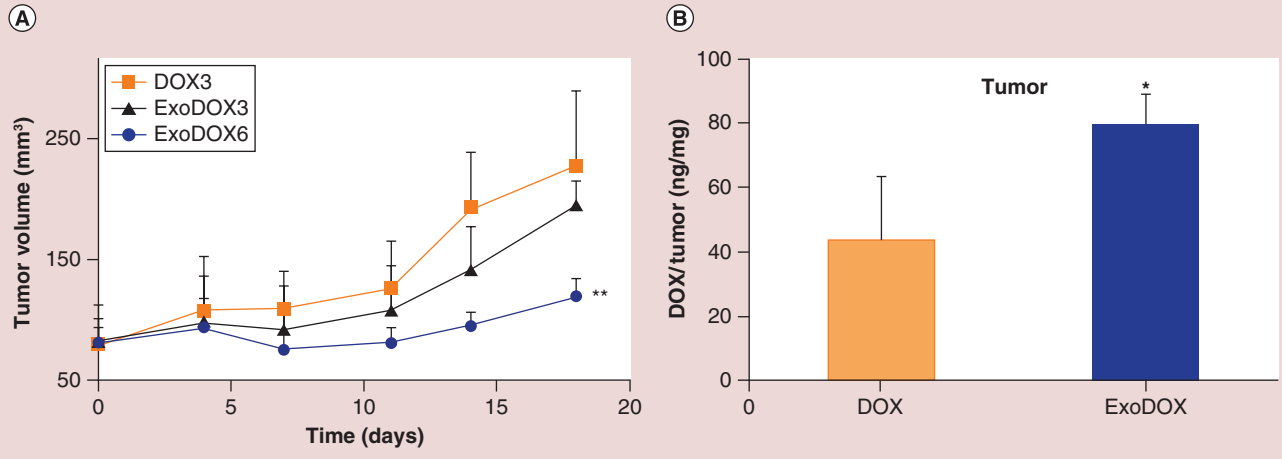


**Figure 6. ExoDOX efficacy on STOSE tumor growth.** **(A)** Tumor volume in FVB/N mice treated by ip. injection with (3 mg/kg) (■) of DOX, (3 mg/kg) (▲) of exoDOX and (•) (6 mg/kg) of exoDOX. **(B)** In the tumors (3 h), exoDOX accumulated more compared with the DOX-treated mice. Y axis: concentration of DOX per gram of tissue.
*p < 0.05.
DOX: Doxorubicin; ip.: Intraperitoneally.

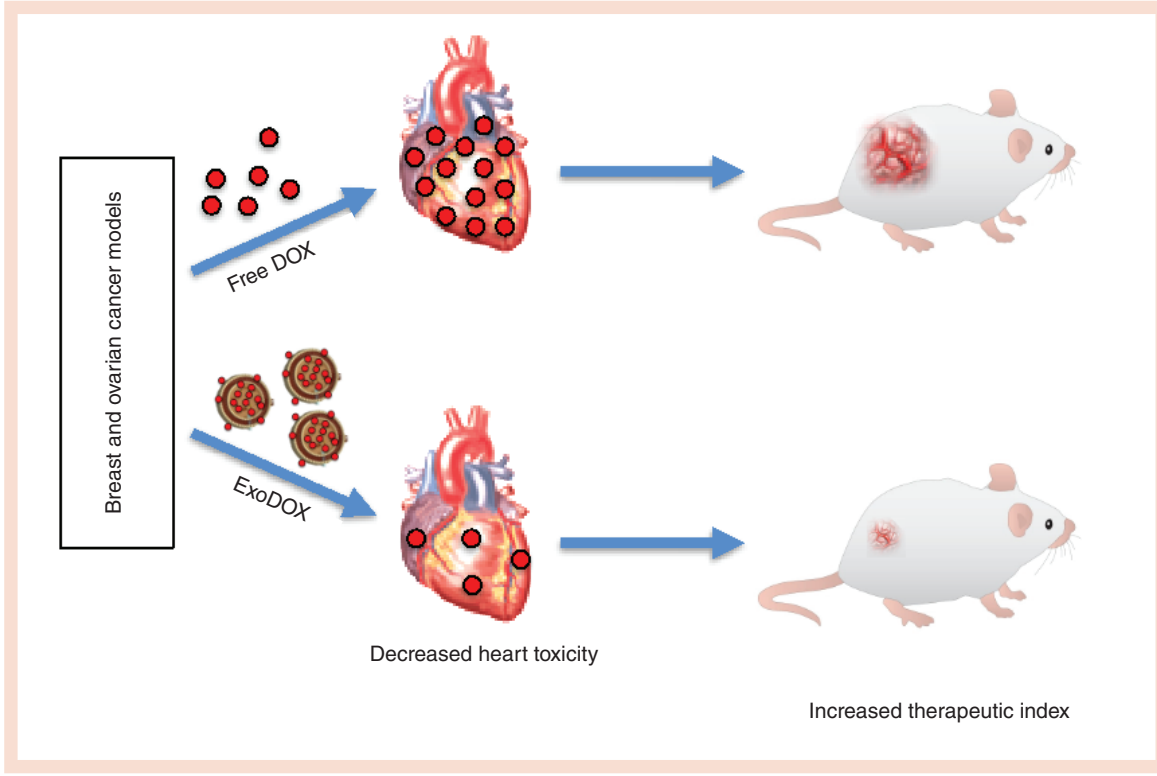


**Figure 7. Working model on the benefit of exosomes as drug delivery system for doxorubicin.**

as exogenous materials in the human body [40].

Exosomes are natural and self-produced vehicles which could transport many types of molecules with targeting ability. It has been demonstrated that melanoma exosomes allow extracellular matrix deposition and vascular proliferation of sentinel lymph nodes to facilitate metastasis [41,42]. Exosomes from breast cancer cells could shuttle to the stroma at metastatic sites in orthotropic nude mouse models [43] and prepare premetastatic stromal cells for tumor cell hosting, utilizing specific miRNAs [44]. Recently, different groups have demonstrated that exosomes can be loaded with DOX and with the aid of targeting moieties can efficiently release DOX to different types of cancer cells [45,46]. Moreover, exosomes can be used to solubilize hydrophobic drugs (e.g., paclitaxel) and increase the activity in regular and multidrug resistance cancer cells [29,30].

Different from other groups, we proved for the first time that exosomes loaded with DOX are less toxic through an altered biodistribution [28]. Physical examination and body weight analyses of mice revealed a better safety profile of exoDOX than DOX avoiding the typical cardiac alterations of DOX. Here, we provide evidences that exosomes can be safely used at a higher dose, thus increasing the therapeutic potential of DOX. We utilized the first spontaneous murine model of HGSOC with defined features such as aneuploidy, gene expression and the presence of an ovarian tumor initiating cell population. As also demonstrated in the breast mouse model, exoDOX are very effective on ovarian cancer cells *in vivo*, thus offering a new therapeutic opportunity for a highly lethal disease.

Clinical trials have demonstrated that exosomes are a biocompatible material that could be safely used in humans. Phase I studies showed no severe adverse effects and set up new clinical grade protocols for the preparation of exosomes. Based on literature and our data, we can envisage an easy application of exosomes as drug delivery system in humans especially for lethal diseases such as cancer.

## Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0154

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## Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

## Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

## Executive summary

**Objective**

- To understand if exosomes may be used to increase the efficacy of doxorubicin (DOX) in breast and ovarian cancers.

**Methods**

- Exosomes were isolated from MDA-MB-231 breast and STOSE ovarian cancer cell lines, and loaded with DOX via electroporation.
- *In vitro* experiments were done to measure the ability of exosomal doxorubicin (exoDOX) to cross the myocardial endothelial cells.
- Toxicity of exoDOX was evaluated in mice by histopathology analysis.
- *In vivo* exoDOX efficacy was derived by measuring tumor volume in breast and ovarian cancer mouse models.
- Biodistribution in mouse tumor tissues of exoDOX was obtained by MS analysis.

**Results**

- ExoDOX partially limits myocardial endothelial cells crossing of DOX.
- DOX encapsulated in exosomes is less toxic and allows to treat mice at a higher concentration.
- When treated with exoDOX compared with free DOX, the volume of breast and ovarian mouse tumors is reduced.
- The concentration of exoDOX in the tumor is higher than that of free DOX.

**Conclusion**

- ExoDOX is safer than DOX in reducing side effects.
- The maximum tolerated dose of exoDOX is higher than free DOX.
- Exosomes increase the therapeutic potential of DOX.
- ExoDOX is a novel alternative therapy for breast and ovarian cancers, both highly lethal diseases.

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