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Research

Cancer cell-derived exosomes as the delivery vehicle of paclitaxel to inhibit cancer cell growth

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Abstract: Delivering water-insoluble drugs, such as paclitaxel (PTX), to the tumor site is a challenge. Exosomes are capable of delivering "cargo" to the target site without causing adverse effects, unlike synthetic nanoparticles. Here we have prepared PTX-loaded exosomes from breast cancer cells. This formulation is further explored for its activity against breast cancer cell lines. Our data indicate that the exosomes are more efficient than free paclitaxel and liposomal paclitaxel in inhibiting the cancer cell growth. The primary reason for this superior activity is determined to be exosomes' ability to deliver more amount of drug in the cytosol. However, the exact mechanism by which exosomes deliver the drug across the cell membrane still needs to be explored.

Keywords: Exosomes, Vesicles, Breast cancer, Cancer therapy, Paclitaxel, Drug delivery

Introduction

Recently, female breast cancer has surpassed lung cancer to become the most commonly diagnosed cancer around the world [1]. Moreover, breast cancer is the fifth leading cause of morbidity in the world [2]. Inefficient drug delivery that involves usage of toxic excipients [3] and untargeted delivery is one of the main reasons for the therapy failure in a number of patients [4,5]. Untargeted delivery results in a significantly less amount of drug accumulating at the target site [5,6]. Moreover, the toxic excipients being used in traditional therapy cause severe health issues in already weak cancer patients [7,8]. Paclitaxel (PTX) is a widely used drug in breast cancer chemotherapy. However, PTX is insoluble in water-based solvents. PTX is administered to patients by dissolving it in a 1:1 mixture of ethanol and Cremophor EL®[9]. The Cremophor EL-based PTX formulation is known to induce much systemic toxicity that includes hypersensitivity [10], anemia [11], skin disorders [12], and peripheral neuropathy [13].

Nanotechnology has tremendously advanced the cancer diagnosis [14-16] and cancer drug delivery regimens [17-20]. Nanoscience provides an alternative targeted-delivery approach that increases the therapeutic index [21] of drugs by reducing excipient-related toxicity and potentially lowers the dose requirements [22-24]. Nanoparticles, by the virtue of their size, get accumulated at tumor sites by a phenomenon known as the enhanced permeability and retention (EPR) effect [25,26]. However, most of the nanoparticles have shown significant organ toxicity and are prone to be cleared by the hepatic and renal systems [27-32].

Recently, there has been lots of interest generated around exosomes [33,34]. Exosomes are ubiquitously present in nature. Exosomes can be isolated from most of bodily fluids such as blood, saliva, and urine. Despite being labeled as

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trash bags [35], exosomes are now proven to be effective communication channels between cells and even between organs [36]. Exosomes carry "information" in the forms of DNA, RNA, and even protein [36,37]. Moreover, exosomes have a size of around 40 to 120 nm, an amicable size for the EPR effect [23]. This makes exosomes an attractive alternative delivery agent for cancer chemotherapeutics. There have been several attempts to develop exosome-based drug-delivery vehicles for the treatment of many chronic diseases, including cancer [38-41]. While exosomes from multiple sources such as stem cells and blood cells are widely studied for their possible application as drug-delivery vehicles, tumor-derived exosomes are not well studied.

In the present work, we developed a novel PTX-encapsulated breast cancer cell-derived exosomes for efficient delivery to breast cancer cells. Our results indicated that exosomal formulation of PTX was more efficient compared to free PTX and even liposomal PTX in inhibiting the growth of breast cancer cells *in vitro*.

Materials and methods

Chemicals and reagents: We used the following reagents and chemicals in our study: Dulbecco's Modified Eagle's Medium (DMEM) (Corning, Manassas, VA, USA); penicillin and streptomycin (Corning, Manassas, VA, USA); fetal bovine serum (FBS) (heat-inactivated) (Cytiva, Marlborough, MA, USA); paclitaxel (PTX) (TCI America, Portland, OR); Total Exosome Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA); SuperSignal™ West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific, Waltham, MA, USA); Trypan blue (Corning, Manassas, VA, USA); Cell Proliferation Reagent WST-1 (Roche, Switzerland); 1,2-dipalmitoyl-sn-glycero-3-phosphotidylcholine (DPPC) (Sigma-Aldrich, St Louis, MO, USA), cholesterol (Sigma-Aldrich, St Louis, MO, USA), chloroform (Sigma-Aldrich, St Louis, MO, USA), phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA).

Cell culture

Breast cancer cell lines (MDA-MB-231 and MCF-7) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in DMEM supplemented with 10% FBS and 200 IU/mL penicillin and 20 µg/mL streptomycin. Cells were maintained as monolayer culture in an incubator maintained at 37 °C temperature and 5% CO2 environment. Tests were performed intermittently to make sure that the cells did not have any mycoplasma contamination.

PTX loading in exosomes and exosome extraction

To extract exosomes from cell culture media, we used the Total Exosome Isolation Kit and followed the manufacturer's protocol. Briefly, we first grew the MDA-MB-231 or MCF-7 cells to confluency, and then the media were replaced with fresh media. After 24 h, the spent media were collected in the centrifuge tubes and reagent from the Total Exosome Isolation kit was added (medium-to-reagent ratio: 2:1). The contents of the centrifuge tube were mixed by gently inverting the centrifuge tube several times until an even solution without any layers was clearly visible. The mixture was kept in a refrigerator at 4 °C overnight. The following day, the contents of the centrifuge were centrifuged at 3000 rpm for 10 min to remove cell debris. The collected supernatant was further centrifuged at 13,000 rpm for 2 h. More than 90% of the top layer of the centrifuged mixture was removed to obtain exosome pellet (often invisible). To obtain PTX-loaded exosomes, MDA-MB-231 cells and MCF-7 cells were treated with 1 µM PTX for 48 h before collecting the spent media. Further, exosomes were collected from the respective spent medium using the Total Exosome Isolation Kit.

Liposome synthesis

PTX-loaded liposomes (Lipo-PTX) were prepared using the thin-film hydration method by partially following a previously reported method [42]. Briefly, DPPC and cholesterol (7:3 molar ratio) were dissolved in 5 mL of chloroform. To this solution PTX was added at a drug-to-lipid ratio of 1:3. The obtained mixture was dried at 35 °C under reduced pressure using a rotary evaporator (Buchi). The resultant lipid cake was hydrated with 2 mL of PBS followed by sonication in an ultrasonic bath at 37 °C for 60 min. To remove the excess PTX, liposome pellet was resuspended in 200 µL of PBS, which was then centrifuged. The obtained pellet was extruded through a membrane with a 400 nm pore size. To prepare liposomes without PTX, a similar protocol was formed without adding PTX.

PTX loading in exosomes

The amount of PTX loaded in exosomes and liposomes was determined by using a protocol reported earlier [23]. Briefly, liposomal or exosomal PTX formulations were airdried, followed by the addition of 20 µL of 8 M urea. The obtained suspension was sonicated in a bath sonicator for 10 min. To this suspension, 60 µL of a solution containing ammonium bicarbonate and tris (2-carboxyethyl) phosphine hydrochloride (50 mM and 10 mM, respectively) and 1.5 µL of trypsin were added. Then the pellets were allowed to be digested for 24 h. The released PTX was quantified by using a reverse-phase high performance liquid chromatography unit equipped with UV detector. A 1:1 mixture of water and acetonitrile was used as mobile phase and diluent. Initially, the peak areas of the PTX pertaining to known standards were recorded by measuring the absorbance at 227 nm. Further, a standard curve was generated by plotting peak area against

concentration. The concentration of PTX in exosomal and liposomal samples was measured by using the same HPLC conditions followed by using the standard curve.

Western blot

Total protein was isolated from exosomes and PTX-loaded exosomes as described earlier [24]. The obtained protein from exosomal samples was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel. After transferring the resolved protein onto a nitrocellulose membrane, CD9 and CD63 proteins were probed against their respective specific antibodies, followed by incubation with HRP-conjugated secondary antibodies. The protein bands were visualized using the West Femto Maximum Sensitivity Substrate kit on a Bio-Rad ChemiDoc imager.

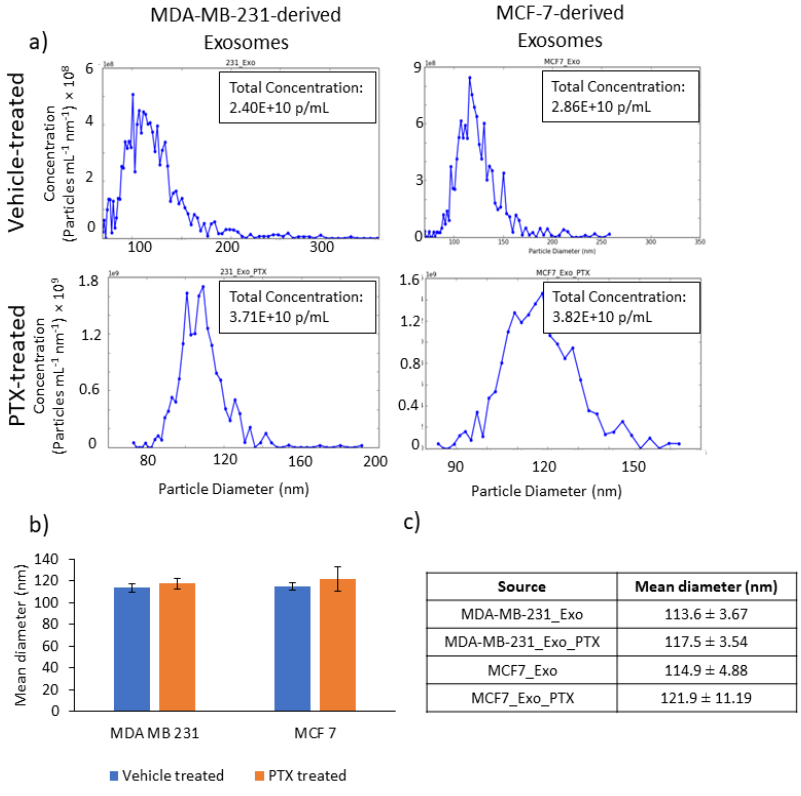
Cell viability assay

To determine the viability of cancer cells (MDA-MB-231) under various conditions, 5×10^3 cells were seeded in a 96-well plate in triplicate. After a 24-h resting period, the cells were treated with free PTX, liposomal PTX, and exosomal PTX (0 to 200 nM). After 48 h cell viability was measured using WST-1 reagent following the manufacturer-recommended protocol as described earlier [43].

Results

Preparation of PTX-loaded exosomes from different cancer cell sources

We aimed to prepare PTX-loaded exosomes to compare its efficiency to that of free PTX and liposomal PTX. To prepare exosomal PTX, we treated confluent flasks of MDA-MB-231 and MCF-7 cells with 1 µM PTX. After 24 h the exosomes were isolated and initially subjected to size analysis using the Spectradyne nCS1 instrument. The size of the exosomes derived from the MDA-MB-231 and MCF-7 cells was in the range of 110 to 125 nm, coinciding with previous reports [44,45] (Figure 1). However, we observed that MCF-7 cells secreted more exosomes compared to MDA-MB-231 cells (Figure 1). We also analyzed the exosomes for the standard exosomal markers CD9 and CD63. Immunoblot analysis revealed that all the isolated exosomes were positive for exosomal marker proteins (Figure 2). It is noteworthy that the drug treatment didn't affect the size, or the surface composition of the exosomes secreted by the cancer cells. Further, the exosomes (and liposomes) were analyzed by HPLC instrument equipped with UV instrument to determine their respective PTX content. The HPLC analysis revealed that MDA-MB-231 cells were more effective in packaging PTX in exosomes (Figure 3). When the amount of PTX packaged in exosomes was normalized to the protein content of the exosomes, the packaging efficiency of MDA-MB-231 cells was ~28% more than that of MCF-7 cells.



Source	Mean diameter (nm)
MDA-MB-231_Exo	113.6 ± 3.67
MDA-MB-231_Exo_PTX	117.5 ± 3.54
MCF7_Exo	114.9 ± 4.88
MCF7_Exo_PTX	121.9 ± 11.19

Figure 1 Exosomes were extracted from MDA-MB-231 cells after treating with Vehicle or PTX. a) Size was measured by Spectradyne nCS1 instrument. b) Data were plotted as a bar graph and also in c) a tabulated form.

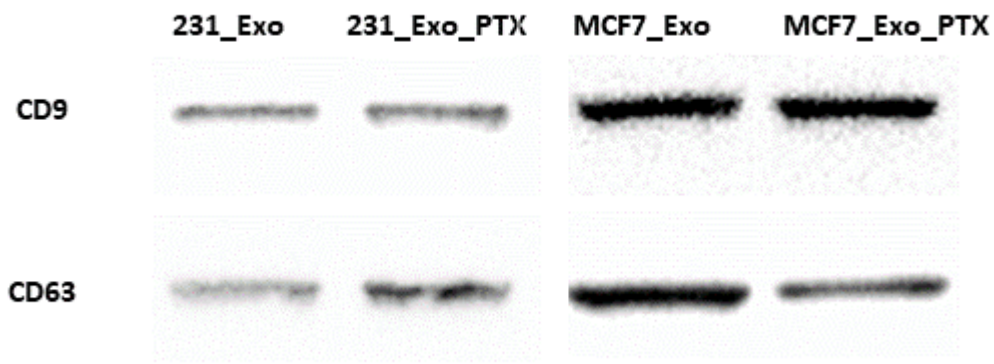


Figure 2 Integrity of the exosomes is confirmed by the presence of CD9 and CD63 markers as determined by immunoblotting.

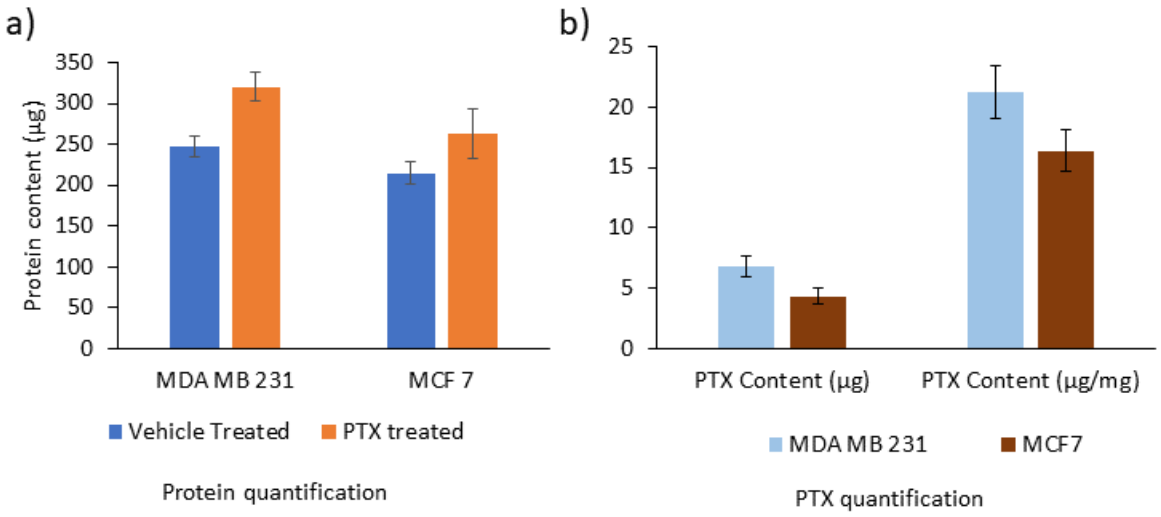


Figure 3 a) Total exosome content was measured using a Micro BCA analysis kit. b) Further, total PTX content in the exosomes was measured by HPLC and normalized to exosomal protein content.

Exosomal PTX outperforms free PTX and liposomal PTX in anti-cancer activity in vitro

PTX is a potent anti-cancer drug that is toxic to all actively dividing cells. To examine whether the encapsulation of PTX in exosomes enhances its efficiency and to compare its activity to that of liposomal and free PTX, MDA-MB-231 and MCF-7 cells were treated with all three formulations (PTX concentration from 0 to 200 nm) separately for 48 h. We used MDA-MB-231 cell-derived exosomal PTX for all the experiments as the PTX payload per microgram of MDA-MB-231 cell-derived exosome was higher than that of MCF-7 cell-derived exosomes. Our data indicated that exosomal PTX is superior in anti-cancer effect against both the tested breast cancer cell lines followed by liposomal formulation and free PTX, respectively (Figure 4). In MBA-MB-231 cells exosomal PTX formulation had an IC_{50} value of 2.87 nM (95% confidence interval (CI) 2.37-3.48 nM), compared to the liposomal-PTX IC_{50} of 8.11 nM (95% CI 7.26-9.10 µM) and the free-PTX IC_{50} of 12.14 nM (95% CI 10.52-14.00 µM). In MCF-7 cells exosomal PTX formulation had an IC_{50} value of 2.36 nM (95% CI 2.01-2.77 µM), compared to the liposomal-PTX IC_{50} of 2.91 nM (95% CI 2.40-3.43 µM) and the free-PTX IC_{50} of 3.83 nM (95% CI 3.08-4.767 µM).

Exosomal PTX is more efficient in inducing apoptosis in triple-negative breast cancer cells

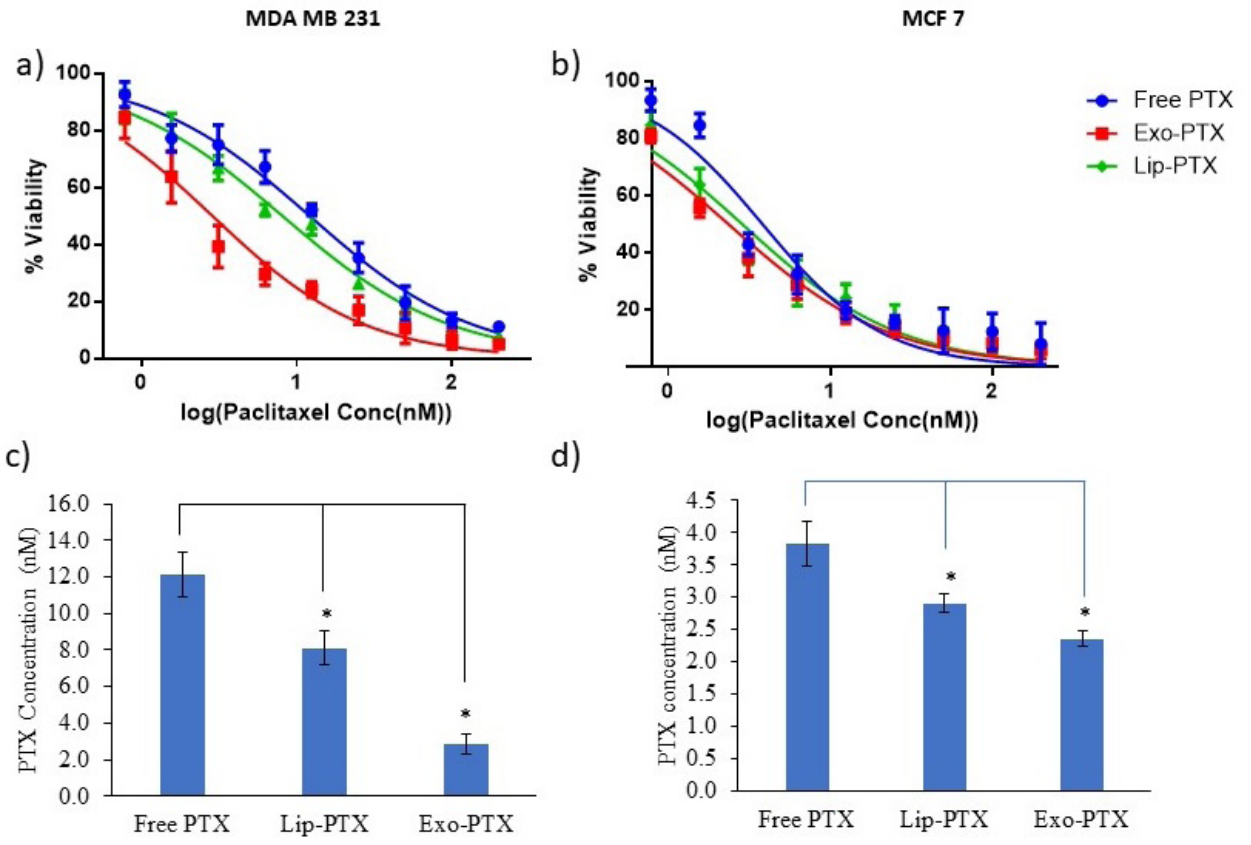


Figure 4 a) MDA-MB-231 cells and b) MCF-7 cells were treated with free PTX, liposomal PTX, and exosomal PTX with concentrations ranging from 0 to 200 nM for 48 h. Cell viability was determined and plotted as the percentage of the control group (0 nM). c) and d) respectively represent the IC_{50} values of the tested PTX formulations against MDA-MB-231 and MCF-7 cells. Data plots were expressed as mean ± standard deviation with n = 3. Statistical analysis was carried out using Student's t-test. *$p < 0.05$ indicates a statistically significant difference.

PTX is known to induce apoptosis in breast cancer cells. To compare the efficiency of the three different formulations of PTX to induce apoptosis in breast cancer cells, we treated MDA-MB-231 cells with equivalent concentrations of exosomal, liposomal, and free formulations of PTX for 48 h. After 48 h, cell lysates were prepared to examine the expression of cell survival-related and apoptosis-related proteins by immunoblotting (Figure 5). We observed that all the formulations induced apoptosis in the cancer cells. However, exosomal formulation of PTX was more efficient in inducing the expression of cyclin-dependent kinase inhibitor, p21, in breast cancer cells compared to all other formulations. Exosomal PTX also increased the expression of a proapoptotic protein, Bax. Further, we observed that treatment with exosomal PTX reduced the expression of cell survival-related proteins Bcl-2 and Bcl-xL more efficiently than all other tested formulations of PTX.

Activity of exosomal PTX is associated with intracellular PTX accumulation

Though it is clear that the exosomal PTX is more efficient in inducing apoptosis in breast cancer cells, the underlying mechanism is still elusive. One possible reason for the enhanced anti-cancer activity of PTX could be due to higher amounts of drug accumulated intracellularly [24]. To verify this assumption, we compared the intracellular accumulation of PTX upon treating with free PTX, liposomal PTX, and exosomal PTX (Figure 6). Initially, breast cancer cells were treated with free PTX, liposomal PTX, and exosomal PTX. After 4 h of treatment, the cells were washed, and cell lysates were prepared. Next, the cell lysates were analyzed for intracellular PTX by HPLC. When the cells were treated with liposomal PTX, the intracellular accumulation of PTX was observed to be 2.75 times higher than that of free PTX treatment. However, the intracellular accumulation was 4.3 times higher when the cells were treated with exosomal PTX compared to free PTX treatment.

Discussion

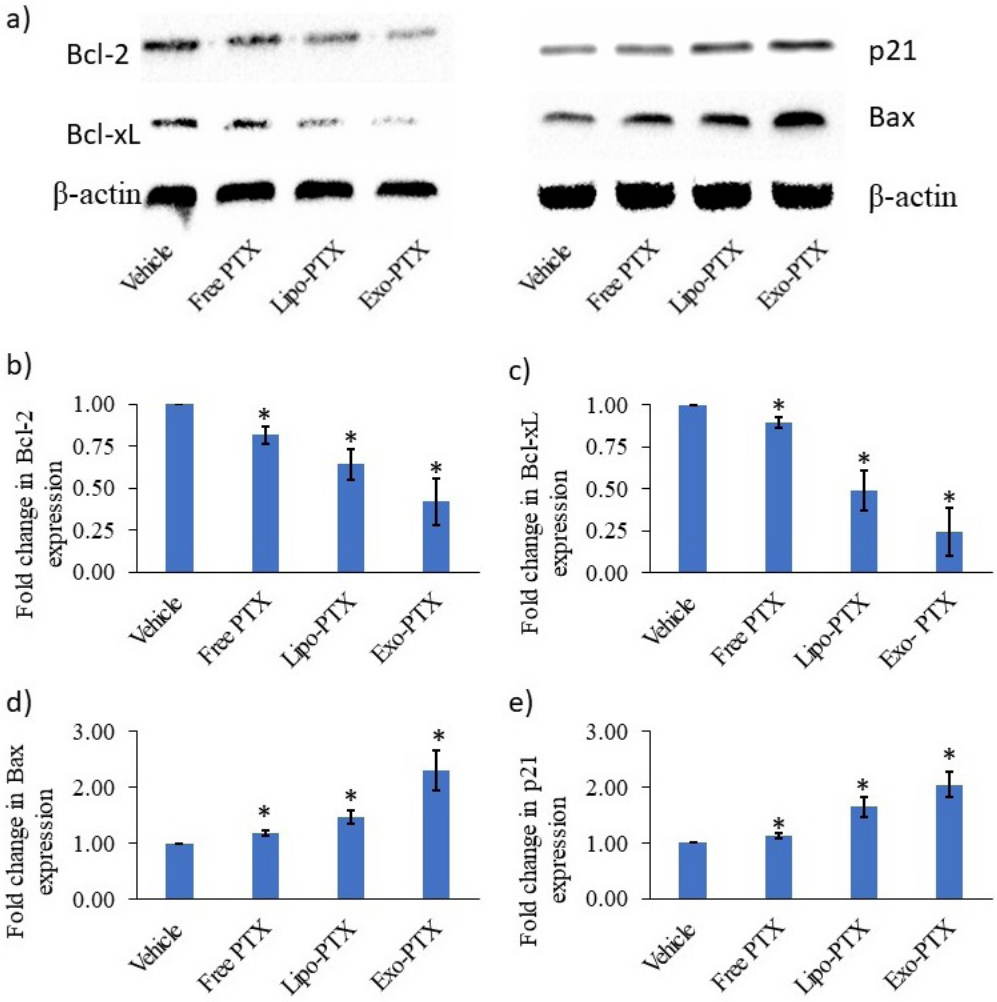


Figure 5 a) MDA-MB-231 breast cancer cells were treated with vehicle, free PTX, Lipo-PTX, and Exo-PTX, and the expression levels of cell survival-related proteins (Bcl-2 and Bcl-xL) and apoptosis-related proteins (p21 and Bax) were studied by immunoblotting. The relative expression of b) Bcl-2, c) Bcl-xL, d) Bax, and e) p21 were quantified by densitometry using Image J software. Data plots were expressed as mean ± standard deviation with n = 3. Statistical analysis was carried out using Student's t-test. *$p < 0.05$ indicates a statistically significant difference.

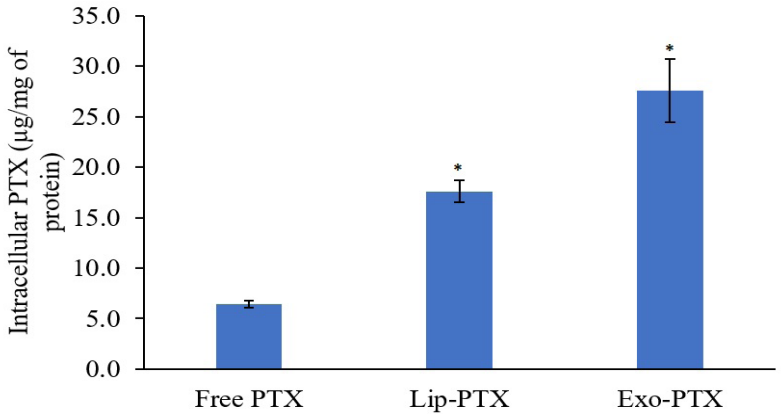


Figure 6 MDA-MB-231 cells were treated with free PTX, liposomal PTX, and exosomal PTX with equivalent concentration of PTX for 4 h. Consequently, the cells were washed, and lysates were prepared to determine the intracellular PTX levels by HPLC. Data plots were expressed as mean ± standard deviation with n = 3. Statistical analysis was carried out using Student's t-test. *$p < 0.05$ indicates a statistically significant difference.

There are multiple challenges that have not yet been addressed in cancer drug delivery. This is one of the main reasons for cancer therapy failure in many cases. Specifically, PTX is an excellent drug but is water-insoluble that leads to usage of toxic organic solvents as excipients. Water-soluble nanoparticles can be used to efficiently deliver PTX to the target site without causing toxic effects. Various metal [46], polymeric [47], and lipid [48] nanoparticles have been proposed as alternative PTX-delivery vehicles. However, these nanoparticles have to cross various biological barriers to deliver the drug to the target site by the EPR effect. However, synthetic nanoparticles face many issues such as generating an immune reaction [31] and/or being cleared by the renal and hepatic systems. Exosomes are naturally occurring nanoparticles that can specifically accumulate at the target site by the virtue of their size and presence of various biological molecules on its surface [49]. Exosomes play an important role in intercellular communication in cancer pathogenesis [50,51] by carrying various biological molecules between cells and organs. All these properties give exosomes a great advantage as drug carrier molecules.

PTX can be loaded into exosomes using various methods as reported earlier [23,24,52]. However, we chose to use the co-incubation method, even though the method is less efficient compared to other methods [53,54], to protect the integrity of exosomes to take full advantage of the amicable surface of the exosomes. Exosomes show a great tropism towards specific cells [55]. To exploit this property, we explored the idea of loading PTX in exosomes secreted by triple-negative breast cancer cells to load PTX and then deliver back to the same cells. To achieve this, we treated breast cancer cells with more than the IC_{50} concentration to ensure the maximum loading of the drug inside the exosomes. The exosomes that were isolated using a commercially available exosome isolation kit showed all the typical exosome characteristics. The exosomes extracted from various sources, vehicle-treated MAD-MB-231 cells, PTX-treated MDA-MB-231 cells, vehicle-treated MCF-7 cells, and PTX-treated MCF-7 cells, showed a size in the range of 110 nm to 130 nm (Figure 1). Further, the presence of CD9 and CD63 markers on the surface of the exosomes, as determined by immunoblotting, established that we were successful in extracting exosomes from various sources. On the other hand, we synthesized free liposomes that didn't contain any PTX as well as PTX-loaded liposomes. The size of the liposomes was established to be about 100 nm as determined by TEM imaging.

Further, we determined the concentrations of PTX in the exosomal (as µg of PTX per mg of exosomal protein) and liposomal (as µg of protein per µL of liposomal suspension) preparations. The amount of PTX encapsulated in the MDA-MB-231-derived exosomes was relatively higher than that in the equivalent amount of MCF-7-derived exosomes. A possible reason for the observation could be that MDA-MB-231 cells are more aggressive in nature (as they can tolerate higher amounts of PTX, an observation deduced from cell viability assay) and therefore are more efficient in eliminating the cytotoxic PTX from their system. As the PTX-treated MDA-MB-231-derived exosomes contain more PTX per µg of protein than PTX-treated MCF-7-derived exosomes, we used the PTX-loaded exosomes derived from MDA-MB-231 cells for all the cell proliferation experiments.

As per our results, free PTX is very toxic to MDA-MB-231 cells (IC_{50} 12.14 nM) and MCF-7 cells (IC_{50} 3.83 nM). However, when PTX was given through liposome nanostructures, there was a great decrease in IC_{50} values. In the case of MDA-MB-231 cells, the IC_{50} decreased to 8.11 nM (a 33.22% drop), and in the case of MCF-7 cells, the IC_{50} decreased to 2.91 nM (a 24.02% drop). This drop in IC_{50} can be attributed to the ability of liposomes to fuse with the cell membrane to efficiently deliver the drug into the cytosol [56]. However, free PTX might not be able to cross the cell membrane with such efficiency. It was even more interesting to observe that when the PTX was delivered in the exosomal form, the IC_{50} further decreased, to 2.87 nM in MDA-MB-231 (a whopping drop of 76.36% compared to free PTX) and to 2.36 nM for MCF-7 cells (a drop of 38.38% compared to free PTX). This huge drop in IC_{50} values can be attributed to various surface proteins that the exosomes carry, which are not present on liposomes' surface [57]. The presence of various proteins and other biological molecules helps the exosomes to anchor onto the surface of the cell membrane, which makes the exosome-cell membrane fusion a lot more efficient. This ultimately leads to more amount of drug being delivered to the cytosol and causes high toxicity to cancer cells. Though the exosomal formulation decreased the IC_{50} values, the degree to which they were decreased was not identical in MDA-MB-231 and MCF-7 cells. One reason could be the tropism of the exosomes. The exosomes derived from MDA-MB-231 cells are preferentially taken up by other MDA-MB-231 cells, leading to the accumulation of more drug in MDA-MB-231 cells. We also observed that the accumulation of more amount of PTX inside the cytosol of cancer cells caused the decrease in the expression of cell survival-related proteins (Bcl-2 and Bcl-xL) and the increase in the expression of apoptosis-related proteins (p21 and Bax). This observation may also be related to exosomes' cell-type specific tropism. However, the specific pathway(s) for the proposed tropism still needs to be established.

To further establish our hypothesis of higher amounts of drug accumulating in the cytosol when the cancer cells are treated with PTX-loaded exosomes, we treated MDA-MB-231 cells with all three different PTX formulations. After treating the cells for a stipulated amount of time, we lysed the cells and quantified the PTX accumulated in them. Our results suggested that when MDA-MB-231 cells were treated with liposomal form of PTX, the total PTX accumulation in cells was observed to be 2.8-fold higher than in the cells treated with free PTX. The accumulation

of PTX was observed to be 4.3-fold higher in the cells treated with PTX-loaded exosomes compared to the cells treated with free PTX.

Conclusion

Finally, our data support our hypothesis that we are successful in loading PTX into exosomes by the simple coincubation method. Our data also establish that exosomal formulation is more potent than free and liposomal formulations of PTX in inhibiting cancer cell growth. The main reason for this enhanced activity is the ability of exosomes to deliver more drug inside the cytosol. However, the exact biological motifs that are responsible for the enhanced activity of PTX-loaded exosomes need to be thoroughly evaluated. The potency of the proposed formulation also needs to be established in the 3D spheroid model and *in-vivo* models before recommending the exosome-based PTX for clinical studies.

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Author contributions

RK conceived and designed the study. RK and CB performed the experiments. RK and CB contributed to acquiring, analyzing, and interpreting the data. RK wrote the manuscript. All the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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