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Doxorubicin and Paclitaxel-loaded Lipid-based Nanoparticles Overcome Multi-Drug Resistance by Inhibiting P-gp and Depleting ATP

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Abstract

To test the ability of nanoparticle (NP) formulations to overcome P-gp-mediated multidrug resistance (MDR), several different doxorubicin (Dox) and paclitaxel (PX)-loaded solid lipid NPs were prepared. Dox NPs showed 6-8-fold lower IC₅₀ values in Pgp overexpressing human cancer cells than those of free Dox. The IC₅₀ value of PX NPs was over 9-fold lower than that of Taxol® in P-gp-overexpressing cells. A series of in-vitro cell assays were used including quantitative studies on uptake and efflux, inhibition of calcein acetoxymethylester (Calcein AM) efflux, alteration of ATP levels, membrane integrity, mitochondrial membrane potential, apoptosis and cytotoxicity. Enhanced uptake and prolonged retention of Dox were observed with NP-based formulations in P-gp-overexpressing cells. Calcein AM and ATP assays confirmed that blank NPs inhibited P-gp and transiently depleted ATP. Intravenous injection of pegylated PX BTM NPs showed marked anticancer efficacy in nude mice bearing resistant NCI/ADR-RES tumors versus all control groups. NPs may be used to both target drug and biological mechanisms to overcome MDR via P-gp inhibition and ATP depletion.

Keywords

anticancer drugs; cancer; polyoxyethylene 20-stearyl ether (Brij 78); endocytosis; ATP

Introduction

Drug resistance is the major cause of failure of cancer chemotherapy. Multi-drug resistance (MDR) is a term used to describe the broad-spectrum resistance to chemotherapy in human cancer, which is a complex phenomenon that can result from several biochemical mechanisms that are still not fully understood (1). A widely studied mechanism of MDR is that resulting from altered cell membrane transport. P-glycoprotein (P-gp) encoded by the *mdr1* gene is well characterized and known to be a clinically important transporter protein belonging to the ATP-binding cassette (ABC) family of membrane transporters (2). It has been shown to pump

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substrates, including doxorubicin and paclitaxel, out of tumor cells through an ATP-dependent mechanism which reduces the effective drug concentrations and consequently decreases the cytotoxic activity. A large number of P-gp inhibitors have been developed. However, clinical trials have been disappointing due to the high inherent toxicities of P-gp inhibitors and/or changed pharmacokinetics and biodistribution properties of anticancer drugs co-administrated with P-gp inhibitors (3).

Formulation strategies have been developed to potentially address P-gp-mediated resistance including colloidal delivery systems, polymer-drug conjugates, and polymeric-micelles. Certain drug-loaded liposomes (4,5) and solid lipid nanoparticles (6,7) have been shown to decrease the resistance of P-gp-expressing cells in-vitro, which has been attributed to increased cellular accumulation of the drug. However, importantly, intracellular drug was still removed by P-gp efflux since formulations did not affect P-gp function (8,9). Anticancer drugs conjugated to polymers such as *N*-(2-hydroxypropyl) methacrylamide (pHPMA) have been shown to effectively kill both sensitive and resistant cancer cells. Proposed mechanisms for these conjugates in resistant cells include internalization by endocytosis, partial inhibition of P-gp gene expression (10), and modification of caspase-dependent apoptosis signaling pathways (11). Polymeric-micelles based on Pluronic[®], co-block polymers comprised of poly(oxyethylene)-poly(oxypropylene), have been used to modulate P-gp in cancer cells (12,13). Pluronic micelles have been utilized to selectively inhibit the P-gp efflux system by ATP depletion in P-gp cells, as well as to reduce the glutathione (GSH) / glutathione S-transferase (GST) detoxification system and to alter apoptotic signal transduction.

Our laboratory has previously developed paclitaxel NPs wherein the drug was entrapped into NPs having emulsifying wax (E. wax) as the oil phase and polyoxyethylene 20-stearyl ether (Brij 78) as the surfactant. These PX NPs were used to overcome P-gp-mediated resistance in-vitro in a human colon adenocarcinoma cell line (HCT-15) (14) and in-vivo in a nude mouse HCT-15 xenograft model (15). The purpose of the present paper was to determine how improved PX NPs and new Dox NPs overcome P-gp-mediated resistance.

Materials and Methods

Tumor cell lines and cell culture

The P-gp-overexpressing human ovarian carcinoma cell line NCI/ADR-RES and sensitive cell line OVCAR-8 were both obtained from National Cancer Institute. The P-gp-overexpressing human melanoma cell line MDA-MB-435/LCC6MDR1 and matching sensitive cell line were kindly provided by Dr. Robert Clarke (Georgetown University).

Preparation and characterization of Dox and PX nanoparticles

Drug-loaded nanoparticles were prepared directly from warm o/w microemulsion precursors. Dox NPs were prepared and characterized as previously described (16). Dox release studies (n=3) were completed at 37°C using the dialysis method. Dox NPs (200 µL) in a cellulose ester dialysis membrane (MWCO: 100,000) were submerged in PBS (pH 7.4) at 37°C. Released Dox was measured by fluorescence detection at 480 nm excitation and 550 nm emission. PX NPs were also prepared and characterized as previously described (17). To prepare pegylated PX BTM NPs, Brij 700 (8% w/w/ ratio of Brij 700 to Miglyol 812) was added before cooling the microemulsion.

In-vitro cytotoxicity studies

Cells were seeded into 96-well plates at 1.5×10^4 cells/well and allowed to attach overnight. Cells were incubated for 48 h with drug equivalent concentrations of all test articles ranging from 10,000 nM to 0.01 nM of free drug (corresponding to 5.45 µg/ml to 5.45 pg/ml for Dox,

and 8.54 $\mu\text{g/ml}$ to 8.54 pg/ml for PX). The sulforhodamine B (SRB) assay was performed and IC50 values were calculated based on the percentage of treatment over control (18).

Cellular uptake and efflux of Dox in cells

Cells were seeded in 48-well plates at a density of 2×10^5 cells/well and incubated overnight. Confluent cell monolayers were washed with Earle's balanced salt solution (EBSS) and treated with test articles. All samples were diluted with EBSS buffer and adjusted to 5 μg Dox/ml or the Dox equivalent concentration. Cells were treated with samples at 37°C for 0.5, 1 and 2 h. Cells were washed twice with ice-cold PBS pH 7.4 and lysed with PBS containing 1% Triton X-100 at 37°C for 30 min. For efflux studies, cells were treated with each sample for 2 h, washed, and then cells were incubated with EBSS buffer at 37°C for another 1, 2 and 4 h. Dox concentrations in cell lysates were measured by HPLC on an Inertsil ODS-3 column with a mobile phase consisting of 0.1 M ammonium formate containing 0.14 % triethylamine (adjusted pH to 2.4 by addition of formic acid)-acetonitrile-methanol-tetrahydrofuran (60:25:17.5:2.5, v/v/v/v) at a flow rate of 1.0 ml/min, and Dox was detected by fluorescence with 480/550 nm excitation/emission. Dox concentrations were normalized for protein content as measured with the BCA assay (Pierce) (19). The cell efflux rate was calculated as: (uptake at 2 h – efflux at 4 h)/4.

Calcein AM assay for P-gp function

A calcein AM assay was performed using a modified method (20). Briefly, cells were seeded in black 96-well plates at a density of 1×10^5 cells/well overnight and treated with 50 μl of various doses of test articles diluted in EBSS buffer. After 0.5 h at 37°C, 50 μl of 0.25 μM calcein AM (Sigma-Aldrich) were added into each well and the fluorescence of calcein was immediately measured every 5 min for 1 h using a microplate reader with 485/589 excitation/emission at room temperature. For pre-treatment experiments, cells were exposed to blank NPs #2 for 0.5 h and washed, and then 50 μl of fresh EBSS buffer was added before addition of calcein AM. The % relative fluorescence in the cells was expressed as: % Relative Fluorescence (FL) = $[(\text{FL}_{\text{treatment}} - \text{FL}_{\text{nontreatment}}) / \text{FL}_{\text{nontreatment}}] \times 100\%$

To assess cell membrane integrity, cells were treated with the same samples for 0.5 h at 37°C, and then incubated for 1 h at room temperature. Then, cells were trypsinized and 50 μl of 0.4 % trypan blue solution was added. Membrane integrity was normalized to untreated control and expressed as (viable cells/total cells) $\times$ 100%.

ATP and Apoptosis assays

An ATP assay was performed as described previously (21). Cells were seeded in 48-well plates at a density of 2×10^5 cells/well and incubated overnight. Various doses of test articles in EBSS buffer were added to cells and incubated at 37°C for 2 h. Following treatment, cells were washed twice with ice-cold PBS and lysed with PBS containing 1% Triton X-100 at 37°C for 30 min. ATP in cell lysates was then measured using ATPlite 1step[®] Assay Kit (PerkinElmer) and normalized for protein content. Cell apoptosis under the tested conditions above was measured using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences).

To assess whether cells could recover from ATP depletion, cells were incubated with various concentrations of test articles for 2 h, washed, and then incubated for another 4 h and 13 h, and then ATP levels and total proteins were measured.

Histology studies by TEM

Cells were washed, scraped, and fixed in 4% buffered formalin, post-fixed in 2% osmium tetroxide, and dehydrated in ascending concentrations of ethanol before embedded in Araldite

502. Blocks were sectioned at 1 micron and 800 Å for light and electron microscopy. Thin sections were stained with lead citrate and uranyl acetate before examined using a Philips 10 electron microscope operated at 60 kV.

Mitochondrial potential measurement

Cells were seeded in black 96-well plates at a density of 1×10^5 cells/well overnight and treated with test articles for 2 h at 37°C. After washing, mitochondrial potential was detected using the JC-1 Mitochondrial Membrane Potential Detection Kit (Biotium). 1.5 or 0.15 µg/ml of PX BTM NPs and 6 µg/ml of cyclosporin A were used as positive and negative controls. Mitochondrial potential was expressed as $(\text{red FL} / \text{green FL})_{\text{treatment}} / (\text{red FL} / \text{green FL})_{\text{nontreatment}} \times 100\%$.

MTT assay

Resistant cells and their sensitive parental cells were seeded in 96-well plates as a density of 4×10^4 overnight and incubated with MTT and samples for 2 h at 37°C, 200 µl of reagent containing 20% SDS and 50% dimethyl formamide in water was added and incubated for 1 h at room temperature and plates were read at 570 nm (test) and 650 nm (reference).

In-vivo anticancer efficacy study using pegylated PX BTM NPs

Female nude (nu/nu) mice, 4–5 weeks (Harlan Laboratories) were housed in a pathogen-free room. All experiments involving the mice were carried with an approved protocol by the University of North Carolina Animal Care and Use Committee. The mice were injected subcutaneously in the interscapular region with 4×10^6 NCI/ADR-RES cells suspended in DMEM medium. When the tumors exhibited volume between 50 – 150 mm³, the mice were randomly assigned to different treatments at two different PX doses of 4.5 mg/kg and 2.25 mg/kg. Mice were injected with either 100 or 200 µL of isotonic treatment article by intravenous injection. Tumors were measured in two perpendicular dimensions every two days for 12 days, and the tumor volume was calculated using the formula: $V = (L \times W^2)/2$, where L and W are the longest and shortest diameters, respectively.

Statistical analysis

Statistical comparisons were made with ANOVA followed by pair-wise comparisons using Student's *t* test using GraphPad Prism software. Results were considered significant at 95% confidence interval ($p < 0.05$).

Results

Dox and PX nanoparticles

The compositions and physicochemical properties of Dox and PX NPs are shown in Table 1A and Table 1B, respectively. PX could be entrapped directly into G78 NPs and BTM NPs. Dox ion-pair complexes with STDC were somewhat soluble in PBS which led to increased rates of Dox release from the NPs. In comparison, STS fully precipitated Dox at a mole ratio of 1:1.2 (Dox: STS) and resulted in an ion-pair complex that had both low solubility in PBS and high solubility in the melted oil phases. All NPs were stable over one month at 4°C (data not shown).

In-vitro cytotoxicity studies

Cytotoxicity data in two pairs of parental (sensitive) and P-gp cell lines are reported in Fig. 1. As expected, in the tested Dox concentration range, free Dox showed no toxicity in NCI/ADR-RES cell line in the tested concentration range ($IC_{50} > 5.45$ µg/ml, corresponding to $> 10,000$ nM) and very low toxicity in MDA-MB-435/LCC6MDR1 cell line ($IC_{50} = 3.62$ µg/ml, corresponding to 6643 nM) (Fig. 1A and 1B). Dox-loaded NPs showed a clear dose-dependent

cytotoxicity against all tested cell lines. In sensitive cell lines, the IC₅₀ values of Dox-loaded NPs were comparable to those of free Dox. In comparison, the IC₅₀ values of Dox NPs #2 were 8-fold lower in NCI/ADR-RES cells (IC₅₀s < 0.61 µg/ml, corresponding to 1111 nM) and in MDA-MB-435/LCC6MDR1 cells (IC₅₀ < 0.45 µg/ml, corresponding to 821 nM) than those of free Dox. Blank NPs did not cause significant cytotoxicity against all cell lines up to a total NP dose of 30 µg/ml.

Interestingly, the post-addition of Dox to blank NPs showed similar cytotoxicity to Dox NPs in both sensitive and resistant cell lines. Thus, to ascertain if this phenomenon was drug specific, PX G78 NPs and PX BTM NPs were tested for cytotoxicity in OVCAR-8 and NCI/ADR-RES cells and compared to Taxol®. As shown in Fig. 1C and Fig. 1D, the IC₅₀ value of Taxol® in NCI/ADR-RES cells was 495-fold greater (IC₅₀= 3.26 µg/ml, corresponding to 3814 nM) than that in sensitive cells (IC₅₀= 0.00658 µg/ml, corresponding to 7.7 nM). Also, the IC₅₀ value of both PX NPs was over 9-fold lower than that of Taxol® in P-gp cells. Both blank NPs did not show significant cytotoxicity in these cell lines. Similar to when free Dox was post-added to blank NPs, the post-addition of free PX to blank G78 NPs or blank BTM NPs had comparable cytotoxicity to that of PX entrapped in NPs. The IC₅₀ values of the post-addition were slightly lower than those of PX NPs in both cell lines; however, the difference was statistically significant ($p < 0.05$) only in the sensitive cells.

Cellular uptake and efflux of Dox

The uptake and efflux of Dox with various formulations containing 5 µg/ml of Dox was examined in both NCI/ADR-RES and MDA-MB-468 cells at different temperatures (Fig. 2). Dox NPs #2 were chosen as the basic NP formulation for these studies. The uptake of Dox was time-dependent except when cells were pre-treated with blank NPs #2. In NCI/ADR-RES cell line at 37°C, NPs led to over a 2-fold increase in the extent of uptake as compared to treatment with free Dox (Fig. 2A). Similarly, all treatments with NP formulations enhanced the retention of Dox. After cells were treated with Dox NPs #2, greater than 15-fold Dox remained in the P-gp cells and the efflux rate was 1.5-fold lower as compared to free Dox after 4 h of efflux. Importantly, the post-addition of Dox to blank NPs #2 also demonstrated enhanced uptake and retention. To eliminate the possibility that Dox was quickly bound to the surface of blank NPs #2, cells were pre-treated with blank NPs #2 and washed before the addition of free Dox. In this treatment, the uptake of Dox was very rapid and reached a maximum within 0.5 h and 7-fold greater Dox was retained in cells compared to free Dox. However, the efflux rate of this treatment (0.19 [Dox](ng)/[protein](µg)/h) was significantly greater than that of free Dox (0.13 [Dox](ng)/[protein](µg)/h) ($p < 0.05$) (Fig. 2A). The uptake of Dox in NCI/ADR-RES cells at 4°C with Dox NPs #2 and free Dox was 24-fold lower and 10-fold lower, respectively, than those at 37°C. Unlike the uptake at 37°C which showed marked differences between NP groups and free Dox, the differences at 4°C were significantly reduced (data not shown). The extent of Dox uptake was also carried out in sensitive MDA-MB-468 cells at 37°C. NP formulations showed greater uptake than free Dox as shown in Fig. 2B. In fact, the Dox NPs #2 very rapidly entered MDA-MB-468 cells and Dox from NPs quickly and extensively localized inside the nuclei of cells by fluorescence microscopy (data not shown).

To confirm that Brij 78 could also enhance Dox uptake and decrease efflux, NCI/ADR-RES cells were pre-treated with various concentrations of Brij 78 (Fig. 2C). At the concentrations of Brij 78 that showed ATP depletion, pre-treatment of cells with Brij 78 led to comparable Dox uptake enhancement and efflux reduction as compared to blank NPs #2 (Fig. 2A).

Calcein AM assay

The ability of blank NPs #2, and the Brij 78 and TPGS surfactants to inhibit P-gp was evaluated using the calcein AM assay in five resistant and sensitive cell lines (Fig. 3). Under all conditions

tested, the trypan blue assay confirmed that there was no significant loss of cell membrane integrity (data not shown). In resistant cells, the fluorescence caused by intracellular calcein significantly increased in a dose-dependent manner either in the presence of blank BTM NPs (data not shown), blank NPs #2 (Fig. 3A and Fig. 3B) or when cells were pre-treated with blank NPs #2 for 0.5 h (data not shown). Brij 78 and TPGS surfactants also led to a dose-dependent increase in calcein fluorescence over 1 h. In contrast, polystyrene NPs did not increase intracellular fluorescence. In stark contrast, no treatments led to increased intracellular fluorescence compared to calcein AM alone in the sensitive MDA-MB-468 cells (data not shown), OVCAR-8 cells (Fig. 3C) and MDA-MB-435/LCC6 cells (Fig. 3D). However, the human melanoma MDA-MB-435/LCC6 cells showed greater permeability as the uptake of calcein AM was higher in these cells.

ATP and Apoptosis Assays

To further understand the mechanisms by which blank NPs and surfactants inhibited P-gp, intracellular ATP levels in cells were measured after exposure to various concentrations of blank NPs #2, Brij 78 and TPGS. In resistant NCI/ADR-RES and MDA-MB-435/LCC6MDR1 cells, ATP levels decreased in a dose dependent manner after treatment with blank BTM NPs, blank NPs #2 and Brij 78 to 40%, 35% and 20% of the initial value, respectively; however, there was no change in ATP levels after treatment with TPGS (Fig. 4A). Importantly, cyclosporin A and polystyrene NPs did not decrease ATP levels in the tested concentration range in NCI/ADR-RES cells. In contrast, over all tested concentrations in sensitive MDA-MB-468 and OVCAR-8 cells, only 0.5 and 1 $\mu\text{g/ml}$ of Brij 78 and blank NPs #2 (Dox equivalent doses) decreased ATP levels to 86% and 65% of the initial value, respectively (data not shown). However, ATP levels decreased in MDA-MB-435/LCC6 cells, which had no significant difference with those in corresponding resistant cells (data not shown). Also, TPGS decreased ATP levels in this sensitive cell line (data not shown). In the presence of 17 $\mu\text{g/ml}$ of cyclosporin A, ATP levels further decreased by an additional 20–40% at each concentration tested (data not shown). Finally, ATP recovery studies showed that after blank NPs #2 and Brij 78 were removed from NCI/ADR-RES cells, cellular ATP levels returned to 100% after 4 h for the lower concentrations tested and were completely restored after 13 h for all concentrations (Fig. 4B).

Under the conditions analogous to the ATP depletion experiments, four concentrations of Dox-equivalent doses (0.5, 1, 2, and 5 $\mu\text{g/ml}$) for the Brij 78 and TPGS surfactants and blank NPs were tested for their ability to induce apoptosis versus control cells at 2 hr in NCI/ADR-RES cells. Only blank NPs at 5 $\mu\text{g/ml}$ showed significance versus control ($p < 0.05$), and these differences were modest (8% versus 6.7% for control).

TEM

MDA-MB-468, OVCAR-8 and NCI/ADR-RES cells are anchorage dependent epitheloid cells. The effects of Dox on these cells appeared similar with the major changes in the degree of nuclear chromatin compaction. In NCI/ADR-RES and OVCAR-8 cells, Dox NPs #2 or blank NPs #2 treatment induced the most severe changes that included cytoplasmic accumulation of multivesicular bodies, chromatin condensation, and varying degree of mitochondrial swelling. After 1 h of incubation, swollen mitochondria were frequently observed in the resistant cells treated with Dox NPs #2 or blank NPs #2 (Fig. 4C). However, this effect was not observed in the sensitive MDA-MB-468 cells. In comparison, Brij 78 also produced mitochondrial swelling in NCI/ADR-RES and OVCAR-8 cells (data not shown). In human melanoma cells, MDA-MB-435/LCC6 and MDA-MB-435/LCC6MDR1, swollen mitochondria were also observed with the treatment of blank NPs #2 or Brij 78 (data not shown).

Mitochondrial potential measurement

Mitochondrial potential changed in all tested cells treated with blank NPs #2 and Brij 78 at the same concentrations that depleted ATP (Fig. 4D). TPGS did not change mitochondrial potential in all cell lines except in MDA-MDB-435/LCC6. All tested samples changed mitochondrial potential in MDA-MB-435/LCC6 cells except the treatment with 0.15 $\mu\text{g/ml}$ of PX BTM NPs.

MTT assay

To a certain extent, all tested samples including blank NPs #2, Brij 78 and TPGS decreased MTT reduction in a dose-dependent manner, except at the lowest concentration of tested samples (0.05 $\mu\text{g/ml}$ of Dox equivalent dose) (data not shown). The minimum value of MTT reduction at 1 $\mu\text{g/ml}$ of Dox equivalent dose was $71 \pm 8\%$ (versus control) and observed in MDA-MB-435/LCC6 cells treated with blank NPs #2 (30 $\mu\text{g/ml}$).

In-vivo anticancer efficacy study

Tumor volume increased with control, Taxol and blank BTM NPs administration at the two PX or PX-equivalent doses tested. In comparison, a marked anticancer effect of the pegylated PX BTM NPs was clearly observed (Fig. 5). The tumor volume in the two tested pegylated PX BTM NPs groups almost did not change during the course of the study. A statistically significant difference of pegylated PX BTM NPs from all other treatments was observed from day 5 and continued to the end of the study. Blank BTM NPs did not show any clinical signs of toxicity even at the highest dose of 210 mg NPs/kg.

Discussion

The objective of the present studies was to investigate the potential of drug-loaded lipid nanoparticles to overcome P-gp-mediated drug resistance, and to elucidate possible mechanisms. The results of the cytotoxicity studies indicate that all tested drug-loaded NPs significantly reduced IC50 values in P-gp-overexpressing ovarian and melanoma cell lines over free drug. Of interest was that the post-addition of free Dox to blank NPs also showed similar IC50 values compared to Dox-loaded NPs (Fig. 1). This is a similar observation as reported by Némati *et al.* in which Dox-loaded polyalkylcyanoacrylate nanoparticles were used to treat sensitive and resistant leucemic murine cells (22). This observation could be the result of two possibilities: 1) due to strong adhesive properties, Dox is adsorbed onto the surface of NPs (23), and 2) blank NPs could affect P-gp and therefore enhance the cytotoxicity of Dox. To test these two possibilities, PX was used since the neutral PX would be less likely to adhere to the slightly negatively-charged NPs. In a similar manner as Dox NPs, free PX post-added to blank NPs showed comparable cytotoxicity as PX-loaded NPs. Temperature-dependent uptake of Dox NPs #2 in NCI/ADR-RES cells indicates an endocytosis pathway of NPs uptake. Obviously, nanoparticles not only enhanced the uptake of Dox but also improved the retention of Dox in resistant cell lines even if Dox was entrapped into NPs or physically present with blank NPs. More importantly, the uptake and retention of Dox increased when cells were pre-treated with blank NPs or free Brij 78 and then free Dox was added (Fig. 2A and Fig. 2C). These results are in contrast to a previous study that showed that the uptake and retention of Dox in MDA-MB-435/LCC6MDR1 cells were not improved when free Dox was added to polymer-lipid hybrid nanoparticles (PLNs) (8). These contrasting studies seem to indicate a different and preferential mechanism of our NPs from the PLNs on Pgp. Taken as a whole, these studies prove that the lipid-based NPs described in the present studies inhibit the function of P-gp. Although a prior study suggested that an ion-pair complex of Dox and polycyanoacrylic acid, a degradation product of polyalkylcyanoacrylate NPs, may increase the intracellular diffusion of Dox and result in increased efficacy of Dox NPs in resistant cells (24), the present study suggested that the ion-pairing agents likely did not affect P-gp directly since the IC50 of PX NPs prepared without ion-pair agents was also low (Fig. 1C and 1D).

According to the data, the factor that was the most influential was the inclusion of the surfactant Brij 78, but not necessarily TPGS. In fact, it has been shown that some surfactants reverse the activity of P-gp and MRP2 (25). In these studies, we showed that both Brij 78 and TPGS were able to increase calcein AM influx in P-g cells, but only Brij 78 was found to deplete ATP. Calcein AM is a non-fluorescent substrate of P-gp that, once in the cells, is irreversibly converted by cytosolic esterase to calcein, a non-permeable and fluorescent molecule. Thus, the increased intracellular fluorescence of calcein when P-gp cells were exposed to lipid-based NPs indicates the inhibition of P-gp function. Moreover, the integrity of membrane confirmed that the inhibition resulted from a selective interaction with P-gp rather than an unspecific membrane alteration which may increase doxorubicin transport in resistant cells. Additionally, the inhibition of P-gp by blank NPs was not related to nanoparticle size since polystyrene nanoparticles having the same particle size had no effect on the intracellular fluorescence (Fig. 3A). Since P-gp efflux is an energy-dependent process, intracellular ATP levels were investigated. The results of the present studies demonstrate that exposure to blank NPs #2 induces a significant decrease in ATP levels in two resistant cells without inducing cell apoptosis. The effect of individual surfactant on ATP levels also was examined. It is clear that Brij 78, not TPGS, decreased ATP levels in resistant cell lines (Fig. 4A). Even at a very low actual concentration of 4.5 $\mu\text{g/ml}$, which is well below the critical micelle concentration (CMC) of Brij 78 (860 $\mu\text{g/ml}$), Brij 78 reduced the ATP levels after 2 h to 78% of the initial value. The ATP levels in sensitive cells responded differently to different cells. ATP levels only slightly changed in MDA-MB-468 and OVCAR-8 cells whereas ATP levels in MDA-MB-435/LCC6 cells decreased to the same extent as with the corresponding resistant cells after treatment with either blank NPs #2 or Brij 78. Our findings on ATP depletion are in agreement with the previous reports from the Kabanov group that concluded that ATP depletion caused by Pluronic[®] P85 block co-polymer was one of the major reasons for reversal P-gp activity and dominant in P-gp cells (20,26). Moreover, Brij 78 had similar influence on enhanced Dox uptake and retention as compared to blank NPs #2 (Fig. 2C). The results of the present study also suggested that reversal of P-gp function by ATP depletion caused by NPs was transient and reversible, based on ATP recovery studies (Fig. 4B) and uptake and efflux studies (Fig. 2A and Fig. 2C).

Our previous studies suggested that Brij 78 could influence the activity of an alcohol dehydrogenase/NAD⁺ enzyme system in-vitro (27). The mitochondria are responsible for regulation of cellular metabolism, and also are the ATP factory in cells. Increase in matrix volume of mitochondria can be due to energetic stress inside cells (28,29). It is important to note that the MTT reduction may not result from cell death since the apoptosis data and ATP recovery data showed that cells did not undergo apoptosis and that the ATP depletion was transient. Therefore, the MTT assay data more likely suggested a change in the cell metabolic activity or enzyme activity in the mitochondrial respiratory chain in treated cells (30,31). Therefore, it is likely that the metabolic activity was decreased in all cells treated with blank NPs #2 and Brij 78. Moreover, mitochondrial potential in resistant and sensitive cell lines changed at the same concentrations of blank NPs #2 or Brij 78 that depleted ATP. However, the change in mitochondrial potential and mitochondrial swelling did not destroy mitochondrial function as ATP levels returned to normal within 4–13 h depending on the dose (Fig. 4B). It is worthy to note that MDA MB-435/LCC6 cells showed both changes in ATP levels and mitochondrial potential. These effects may be related to the high permeability of the cell membrane as observed by the relatively high uptake of calcein AM (Fig. 3D). The change in the mitochondrial potential was correlated with mitochondrial swelling in all tested cells in these studies. Thus, it is possible that Brij 78 and NPs influence the enzymes involved in mitochondrial respiratory chain, and consequently produce energy stress in cells. As a consequence, the mitochondrial potential changes and the mitochondria swell to meet the energy requirement. These effects are likely pronounced in Pgp-overexpressing cells as they require more energy for P-gp expression and function.

The current study suggested that there are at least two major reasons for enhanced cytotoxicity of Dox or PX-loaded lipid-based NPs in P-gp-mediated resistant cells: 1) increased extent of drug uptake by endocytosis of NPs which helps to partially bypass P-gp; 2) decreased efflux rate of drug through inhibition of P-gp function and ATP depletion caused by Brij 78, a component of NPs. Both increase intracellular drug concentrations which is the key to overcoming transporter mediated resistance. In-vivo anticancer efficacy in mice bearing resistant NCI/ADR-RES cell xenografts demonstrated that pegylated PX BTM NPs significantly inhibited tumor growth versus all tested controls. The BTM NPs also showed the ability to inhibit P-gp function and deplete ATP. Additional in-vivo studies are on-going including pharmacokinetic and biodistribution studies, as well as additional efficacy studies in resistant and sensitive tumor mouse tumor models.

In conclusion, both Dox and PX-loaded lipid-based NPs containing the Brij 78 surfactant were shown to overcome P-gp-mediated drug resistance. The mechanism of Pgp inhibition and ATP depletion distinguishes these Brij 78-based NPs from other known nanoparticles and liposome-based carrier systems. To the best of our knowledge, this is the first report on nanoparticles which can inhibit P-gp efflux and deplete ATP. Most importantly, NP-based carriers that effectively target both the drug and biological mechanisms to overcome MDR (P-gp inhibition and ATP depletion) appear to be a novel therapeutic strategy and additional *in vivo* work is warranted.

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References

1. Volm M, Mattern J. Resistance mechanisms and their regulation in lung cancer. *Crit Rev Oncog* 1996;7:227–244. [PubMed: 9258604]
2. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002;2:48–58. [PubMed: 11902585]
3. Wishart GC, Bissett D, Paul J, et al. Quinidine as a resistance modulator of epirubicin in advanced breast cancer: mature results of a placebo-controlled randomized trial. *J Clin Oncol* 1994;12:1771–1777. [PubMed: 8083699]
4. Rahman A, Husain SR, Siddiqui J, et al. Liposome-mediated modulation of multidrug resistance in human HL-60 leukemia cells. *J Natl Cancer Inst* 1992;84:1909–1915. [PubMed: 1361008]
5. Booser DJ, Esteva FJ, Rivera E, et al. Phase II study of liposomal doxorubicin in the treatment of doxorubicin-resistant breast cancer. *Cancer Chemother Pharmacol* 2002;50:6–8. [PubMed: 12111105]
6. Soma CE, Dubernet C, Barratt G, et al. Ability of doxorubicin-loaded nanoparticles to overcome multidrug resistance of tumor cells after their capture by macrophages. *Pharm Res* 1999;16:1710–1716. [PubMed: 10571276]
7. Wong HL, Rauth AM, Bendayan R, et al. A new polymer-lipid hybrid nanoparticle system increases cytotoxicity of doxorubicin against multidrug-resistant human breast cancer cells. *Pharm Res* 2006;23:1574–1585. [PubMed: 16786442]
8. Wong HL, Bendayan R, Rauth AM, Xue HY, Babakhanian K, Wu XY. A mechanistic study of enhanced doxorubicin uptake and retention in multidrug resistant breast cancer cells using a polymer-lipid hybrid nanoparticle system. *J Pharmacol Exp Ther* 2006;317:1372–1381. [PubMed: 16547167]
9. Chavanpatil MD, Patil Y, Panyam J. Susceptibility of nanoparticle-encapsulated paclitaxel to P-glycoprotein-mediated drug efflux. *Int J Pharm* 2006;320:150–156. [PubMed: 16713148]
10. Minko T, Kopeckova P, Pozharov V, Kopecek J. HPMA copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line. *J Control Release* 1998;54:223–233. [PubMed: 9724909]

11. Minko T, Kopeckova P, Kopecek J. Preliminary evaluation of caspases-dependent apoptosis signaling pathways of free and HPMA copolymer-bound doxorubicin in human ovarian carcinoma cells. *J Control Release* 2001;71:227–237. [PubMed: 11295216]
12. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic block copolymers for overcoming drug resistance in cancer. *Adv Drug Deliv Rev* 2002;54:759–779. [PubMed: 12204601]
13. Minko T, Batrakova EV, Li S, et al. Pluronic block copolymers alter apoptotic signal transduction of doxorubicin in drug-resistant cancer cells. *J Control Release* 2005;105:269–278. [PubMed: 15939500]
14. Koziara JM, Lockman PR, Allen DD, Mumper RJ. Paclitaxel nanoparticles for the potential treatment of brain tumors. *J Control Release* 2004;99:259–269. [PubMed: 15380635]
15. Koziara JM, Whisman TR, Tseng MT, Mumper RJ. In-vivo efficacy of novel paclitaxel nanoparticles in paclitaxel-resistant human colorectal tumors. *J Control Release* 2006;112:312–319. [PubMed: 16626835]
16. Ma P, Dong X, Swadley CS, Gupte A, Leggas M, Ledebur HC, et al. Development of idarubicin and doxorubicin solid lipid nanoparticles to overcome Pgp-mediated multiple drug resistance in leukemia. *J Biomed Nanotechnol.* 2009January on-line publication
17. Dong X, Mattingly CA, Tseng MT, Cho M, Adams VR, Mumper RJ. Development of new lipid-based paclitaxel nanoparticles using sequential simplex optimization. *Eur J Pharm Biopharm.* 2008 December 10;
18. Papazisis KT, Geromichalos GD, Dimitriadis KA, Kortsaris AH. Optimization of the sulforhodamine B colorimetric assay. *J Immunol Methods* 1997;208:151–158. [PubMed: 9433470]
19. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76–85. [PubMed: 3843705]
20. Polli JW, Wring SA, Humphreys JE, et al. Rational use of in vitro P-glycoprotein assays in drug discovery. *J Pharmacol Exp Ther* 2001;299:620–628. [PubMed: 11602674]
21. Batrakova EV, Li S, Elmquist WF, Miller DW, Alakhov VY, Kabanov AV. Mechanism of sensitization of MDR cancer cells by Pluronic block copolymers: Selective energy depletion. *Br J Cancer* 2001;85:1987–1997. [PubMed: 11747344]
22. Némati F, Dubernet C, de Verdière AC, et al. Some parameters influencing cytotoxicity of free doxorubicin and doxorubicin-loaded nanoparticles in sensitive and multidrug resistant leucemic murine cells: incubation time, number of nanoparticles per cell. *Int J Pharm* 1994;102:55–62.
23. Tomlinson E, Malspeis L. Concomitant adsorption and stability of some anthracycline antibiotics. *J Pharm Sci* 1982;71:1121–1125. [PubMed: 6958860]
24. de Verdière AC, Dubernet C, Némati F, et al. Reversion of multidrug resistance with polyalkylcyanoacrylate nanoparticles: towards a mechanism of action. *Br J Cancer* 1997;76:198–205. [PubMed: 9231919]
25. Bogman K, Erne-Brand F, Alsenz J, Drewe J. The role of surfactants in the reversal of active transport mediated by multidrug resistance proteins. *J Pharm Sci* 2003;92:1250–1261. [PubMed: 12761814]
26. Kabanov AV, Batrakova EV, Alakhov VY. An essential relationship between ATP depletion and chemosensitizing activity of Pluronic block copolymers. *J Control Release* 2003;91:75–83. [PubMed: 12932639]
27. Dong X, Mumper RJ. The metabolism of fatty alcohols in lipid nanoparticles by alcohol dehydrogenase. *Drug Dev Ind Pharm* 2006;32:973–980. [PubMed: 16954110]
28. Kaasik A, Safiulina D, Zharkovsky A, Veksler V. Regulation of mitochondrial matrix volume. *Am J Physiol Cell Physiol* 2007;292:C157–C163. [PubMed: 16870828]
29. Halestrap AP. The regulation of the oxidation of fatty acids and other substrates in rat heart mitochondria by changes in matrix volume induced by osmotic strength, valinomycin and Ca²⁺. *Biochem J* 1987;244:159–164. [PubMed: 3663110]
30. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* 2005;11:127–152. [PubMed: 16216776]
31. Stowe R, Koenig DW, Mishra SK, Pierson DL. Nondestructive and continuous spectrophotometric measurement of cell respiration using a tetrazolium-formazan microemulsion. *J Microbial Methods* 1995;22:283–292.

Figure 1.

Dox and PX cytotoxicity studies at 48 h. IC₅₀ values (μg/ml) of Dox and Dox NPs in (A) OVCAR-8 and NCI/ADR-RES cells, and (B) MDA-MB-435/LCC6 and MDA-MB-435/LCC6MDR1 cells. (C) IC₅₀ values (μg/ml) of PX and PX G78 NPs in OVCAR-8 and NCI/ADR-RES cells. (D) IC₅₀ values (μg/ml) of PX and PX BTM NPs in OVCAR-8 and NCI/ADR-RES cells. Data are presented as the mean ± SD of three independent experiments (N=3) with triplicate (n=3) measurements for each experiment. * $p < 0.05$; # and ## $p > 0.05$. “Drug equivalent dose” of NPs and excipients are calculated from the composition shown in Table 1.

A

B

C

Figure 2.

Cellular uptake and efflux of Dox in NCI/ADR-RES and MDA-MB-468 cells. (A) uptake and efflux in NCI/ADR-RES cells at 37°C, and (B) uptake in MDA-MB-468 cells at 37°C. For pre-treatment experiments, cells were pre-treated with blank NPs #2 for 0.5 h before the next step. For efflux studies, cells were treated with samples for 2 h, and then re-incubated with fresh EBSS buffer for indicated time. (C) Dox uptake and efflux when cells were pre-treated with Brij 78 at concentrations of 45.3, 18.1, and 9.1 µg/ml (corresponding to Dox-equivalent doses of 5, 2, and 1 µg/ml, respectively) for 0.5 h. Data are presented as the mean ± SD (n = 3). * $p < 0.05$ and # $p > 0.05$.

Figure 3.

Dose response of blank NPs #2 and surfactants in calcein AM assay in NCI/ADR-RES cells (**A**), MDA-MB-435/LCC6MDR1 cells (**B**), OVCAR-8 cells (**C**), and MDA-MB-435/LCC6 cells (**D**). Concentrations are presented as concentrations of blank NPs #2 (sum of mass of oils and surfactants) and as concentrations of free Brij 78 and TPGS. Data are presented as the mean $\pm$ SD ($n = 3$).

Figure 4.

The effects of nanoparticles on ATP levels and mitochondria. **(A)** Effect of blank NPs #2 and surfactants on intracellular ATP level in NCI/ADR-RES cells and MDA-MB-435/LCC6MDR1 cells. **(B)** ATP recovery in NCI/ADR-RES cells for 4 h and 13 h. Cells were treated with test samples for 2 h at 37°C and then samples were removed from cells. Cellular ATP levels were measured after cells were continuously incubated in fresh medium for another 4 h and 13 h. Concentrations are presented as concentrations of blank NPs #2 (sum of mass of oils and surfactants) and as concentrations of free Brij 78 and TPGS. Data are presented as the mean $\pm$ SD (n = 3). *. * p < 0.05 and # p > 0.05. **(C)** TEM analysis in NCI/ADR-RES, OVCAR-8 and MDA-MB-468 cells treated with Dox NP #2, free Dox, blank NP #2. Cells were treated for 1 h with samples containing Dox equivalent dose at 5 $\mu\text{g/ml}$. Treatment with Dox NPs #2, blank NP #2 caused considerable mitochondria swelling in NCI/ADR-RES and OVCAR-8 cells, but not in MDA-MB-468 cells. The stars (*) indicate multivesicular bodies, and the

arrows (→) indicates mitochondria. Scale bar = 1. **(D)** Mitochondrial potential measurement in NCI/ADR-RES cells and MDA-MB-435/LCC6MDR1 cells and their corresponding parental cells. Cells were treated with samples for 2h at 37°C. Mitochondrial potential detection was based on the ratio of red fluorescence caused by the accumulation of JC-1 in mitochondria and green fluorescence caused by the accumulation of JC-1 in the cytoplasm. The number before each label on x-axle indicates the concentration of tested samples (µg/ml).

Figure 5.

In-vivo anticancer efficacy studies using pegylated PX BTM NPs in resistant mouse NCI/ADR-RES xenografts. On Day (-7), 18–19 g female nude mice received 4×10^6 cells by s.c. injection. Mice (n=4/group) were dosed i.v. with PX (4.5 or 2.25 mg/kg) by tail vein injection on day 0 and 7. The corresponding nanoparticle dose was 210 or 105 mg NPs/kg, respectively. Data are presented as the mean $\pm$ SD

Table 1

Table 1A. Compositions of Dox and PX NPs							
Formulations	Ion-pair	Oil phases (mg/ml)			Surfactants (mg/ml)		
		E. wax	Stearyl alcohol	Glyceryl tridodecanoate	Miglyol 812	TPGS	Brij 78
Dox NPs #1	STDC	2.0	-	-	-	2.6	2.0
Dox NPs #2	STS	2.0	-	-	-	2.6	2.0
Dox NPs #3	STS	2.0	-	-	-	-	4.0
Dox NPs #4	STS	-	2.0	-	-	-	4.0
PX G78 NPs	none	-	-	1.7	-	-	4.0
PX BTM NPs	none	-	-	-	2.5	1.5	3.5

Table 1B. Summary of the physicochemical properties of DOX NPs and PX NPs				
Formulations	Mean diameter (nm)	% Drug loading (w/w, drug/oil)	% Drug entrapment efficiency	% Drug released in 2 h
Dox NPs#1	102.3 ±3.9	10	87.2	77.0 ± 1.1
Dox NPs #2	103.3 ±1.2	15	86.2 ±5.9	45.5 ±7.1
Dox NPs #3	84.9 ±3.3	15	91.0 ±5.9	56.9 ±2.4
Dox NPs #4	68.8 ±2.5	15	91.8 ±4.6	52.8 ±2.4
PX G78 NPs	138.7 ±1.3	7.5	85.4 ±3.3	5.5 ± 1.1
PX BTM NPs	177.3 ± 1.4	6	97.5 ±2.6	8.7 ±4.0

Final concentrations of Dox and PX in drug-loaded NPs were 300 µg/ml and 150 µg/ml, respectively.