Doxorubicin and Lapatinib Combination Nanomedicine for Treating Resistant Breast Cancer

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ABSTRACT: Our objective was to design a polymeric micelle-based doxorubicin and lapatinib combination therapy for treating multidrug resistant (MDR) breast cancers. Poly(ethylene glycol)-block-poly(2-methylen-benzoxycarbonylpropylene carbonate) (PEG-PBC) polymers were synthesized for preparing doxorubicin and lapatinib loaded micelles using a film dispersion method. Micelles were characterized by determining critical micelle concentration (CMC), particle size distribution, and drug loading. The anticancer effects were determined in vitro with MTT assays as well as with lactate dehydrogenase (LDH) release studies. In addition, the cellular uptake of drug-loaded micelles was determined with fluorescence microscopy and flow cytometry. Finally, in vivo anticancer activity and tolerance of developed formulations were evaluated in resistant breast tumor bearing mice. PEG5k-PBC7k polymer synthesized in this study had a low CMC value (1.5 mg/L) indicating an excellent dynamic stability. PEG-PBC micelles could efficiently load both doxorubicin and lapatinib drugs with a loading density of 21% and 8.4%, respectively. The mean particle size of these micelles was 100 nm and was not affected by drug loading. The use of lapatinib as an adjuvant sensitized drug resistant MCF-7/ADR cells to doxorubicin treatment. Cellular uptake studies showed enhanced doxorubicin accumulation in MCF-7/ADR cells in the presence of lapatinib. The doxorubicin and lapatinib combination therapy showed a significant decrease in tumor growth compared to doxorubicin monotherapy. In conclusion, we have developed PEG-PBC micelle formulations for the delivery of doxorubicin and lapatinib. The combination therapy of doxorubicin plus lapatinib has a great potential for treating MDR breast cancer.

KEYWORDS: polymeric micelles, breast cancer, multidrug resistance, combination therapy, lapatinib, doxorubicin, nanomedicine

1. INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer related death in women in the United States, with an estimated 232,340 new cases of invasive breast cancer and 39,620 deaths in 2013.1 Chemotherapy is an important treatment method among currently available therapeutic approaches. Anthracyclines and taxanes are the first-line chemotherapeutic agents for advanced metastatic breast cancer.2,3 However, the use of these agents inevitably induces drug resistance and results in the relapse of breast cancer. Breast cancer develops chemoresistance via multiple mechanisms including overexpression of MDR transporters, defective cell apoptosis, and the presence of cancer stem cells (CSCs).4,5 MDR transporter overexpression is primarily responsible for drug resistance and eventually leads to the failure of breast cancer therapy. P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multiple drug resistance protein (MRPs) are major MDR transporters which increase drug efflux and reduce drug accumulation in tumor cells.5 A number of clinical studies have demonstrated that chemotherapy could induce overexpression of MDR transporters in breast cancers and MDR transporter overexpression correlated with a worse response to the treatment.6–8 Due to the lack of effective clinical interventions, patients with resistant breast cancers have a poor prognosis. Thus, there is an urgent need for effective therapies to overcome MDR and treat resistant breast cancer.

Inhibitors of drug efflux pump have been investigated in clinical trials for MDR reversal and considered as a promising solution to overcome MDR in cancers.9,10 However, a formidable obstacle to clinical success is that the dose of inhibitors for reversing MDR usually causes unacceptable side toxicity, because high concentrations are needed to effectively inhibit MDR transporters. Lapatinib (LAPA) is a dual kinase inhibitor against epidermal growth factor receptor (EGFR) and human epidermal receptor two (HER-2). It has been approved by the FDA since 2007 for use in combination with capecitabine to treat HER-2 positive refractory breast cancers which have progressed following previous chemotherapy with
anthracyclines, taxanes, and trastuzumab (Herceptin).\textsuperscript{11,12} In addition to the activities as an EGFR and HER-2 inhibitor, lapatinib has also shown inhibitory activities on MDR transporters.\textsuperscript{13−15} Lapatinib inhibits MDR transporters through a direct interaction with the substrate-binding site and has been reported for its effectiveness to overcome MDR.\textsuperscript{16−18} Most importantly, the safety of lapatinib has already been well-documented. Its recommended daily dose is 1500 mg for metastatic breast cancer. Therefore, lapatinib may be an ideal drug efflux inhibitor not only for its good safety at a high dose but also for its intrinsic therapeutic effect targeting multiple anticancer mechanisms. In our previous studies, we have used lapatinib and paclitaxel combination therapy to effectively treat resistant prostate cancers.\textsuperscript{18} Being encouraged by these previous studies, we have extended our research and developed a combination therapy using doxorubicin (DOX) and lapatinib to treat MDR breast cancers.

In the current study, we developed a polymeric micelle for improved delivery of the poorly water-soluble doxorubicin and lapatinib. A poly(ethylene glycol)-block-poly(2-methyl-2-benzyloxycarbonylpropylene carbonate) (PEG-PBC) copolymer was synthesized for preparing polymeric micelle formulations. Polymeric micelle is a promising drug delivery carrier that is suitable for the delivery of hydrophobic anticancer drugs, as demonstrated in our or other previous studies.\textsuperscript{19−26} Poly(ethylene glycol) (PEG)-based amphiphilic block copolymers self-assemble in aqueous solution to form core−shell structured polymeric micelles. The hydrophilic PEG block forms a hydrophilic shell surrounding the micelle. It provides an effective steric stabilization and prevents the uptake of micelles by reticuloendothelial system (RES).\textsuperscript{27} The hydrophobic block forms a hydrophobic core which serves as a drug loading reservoir. Amphiphilic polymers assemble into micelles and load hydrophobic drugs through hydrophobic interaction, hydrogen binding, and electrostatic interaction.\textsuperscript{28−31} Polymeric micelles can efficiently encapsulate hydrophobic drugs and significantly improve the drug solubility. The poor solubility of anticancer drugs is a big challenge for their delivery and formulation. Polymeric micelles can effectively address this issue. Because of the small particle size and long circulation in blood, polymeric micelles can specifically deliver drugs into solid tumors through the enhanced permeability and retention (EPR) effects (passive tumor targeting).\textsuperscript{32−34} Therefore, we used polymeric micelles for enhanced delivery of hydrophobic lapatinib and doxorubicin for treating MDR breast cancer (Scheme 1). Formulations were optimized and characterized. Then, both in vitro assays and in vivo studies were performed to evaluate anticancer activities of developed formulations on MDR breast cancer cells.

2. MATERIALS AND METHODS

2.1. Materials. Doxorubicin and lapatinib were purchased from Melone Pharmaceutical Co., Ltd., China. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), methoxypoly(ethylene glycol) (mPEG, \(M_n = 5000\), PDI = 1.03), and other reagents were obtained from Sigma Aldrich and used without further purification. 2-Methyl-2-benzyloxycarbonyl propylene carbonate (MBC) was synthesized as described.\textsuperscript{35}

2.2. Synthesis of Poly(ethylene glycol)-block-Poly(2-methyl-2-benzyloxycarbonyl propylene carbonate) (PEG-PBC). PEG-PBC polymer was synthesized as previously described.\textsuperscript{20,36} Briefly, DBU (40 \(\mu\)L) was added to the mixture of given amount of mPEG and MBC in 10 mL of anhydrous CH\(_2\)Cl\(_2\) and reacted under stirring for 3 h. The reaction mixture was then dissolved in CH\(_2\)Cl\(_2\) precipitated with a large amount of ice-cold diethyl ether, and dried under vacuum at room temperature. Polymers were characterized by \(^1\)H nuclear magnetic resonance (NMR) with a 400 MHz JEOL ECS NMR and deuterated chloroform (CDCl\(_3\)) as a solvent. The molecular weight and polydispersity index (PDI) of PEG-PBC polymers were determined by gel permeation chromatography (GPC) using a Waters GPC system equipped with a GPC column (Styrage HR 4E) and a differential refractive index detector. Tetrahydrofuran was used as an eluent at a flow rate of 1 mL/min. The polymers were characterized by NMR and GPC, and the molecular weight and polydispersity index were determined. The polymers were found to be monodispersed with narrow dispersity (PDI < 1.1). The molecular weight of the polymers was found to be in the range of 30,000−50,000. The polymers were stable at room temperature for several months.

Scheme 1. Schematic Illustration of the Doxorubicin and Lapatinib Combination Nanomedicine for Treating Resistant Breast Cancer

![Scheme 1](https://example.com/scheme1.png)

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of 0.35 mL/min. A series of narrow polystyrene standards (50–100 000 Da) were used for calibration. The critical micelle concentration (CMC) was determined using pyrene as a hydrophobic fluorescent probe. Briefly, 40 μL of pyrene stock solution (2.4 × 10−8 M) in acetone was added to 40 mL water to prepare a saturated pyrene aqueous solution. Polymer samples were dispersed in water with concentration ranges from 0.5 to 4.8 × 10−7 mg/mL and mixed thoroughly with above pyrene solution at a volume ratio of 1:1. The fluorescent intensity was recorded with a fluorescence spectrometer with Fc = 338 nm (I3) and 333 (I1) and Em = 390 nm. The intensity ratio (I3/I1) was plotted against the logarithm of polymer concentration. The CMC value was obtained as the point of intersection of two tangents drawn to the curve at high and low concentrations, respectively.

2.3. Preparation and Characterization of Micelles. 2.3.1. Preparation of Micelles. Polymeric micelles were prepared with a film dispersion method as previously reported with some modifications. Briefly, 15 mg of polymer and a given amount of doxorubicin or lapatinib were dissolved in 0.5 mL of CHCl3, and then the solvent was removed under reduced pressure. The resulting film was hydrated in 3 mL of PBS (pH 7.4) and sonicated for 1 min. Then, the residue free drug was removed by centrifugation at 10 000 rpm for 5 min. The supernatant was filtered using a 0.22 μm filter. For the lyophilization, micelles were frozen overnight at −80 °C and lyophilized with a freeze-dry system (LABCONCO, USA).

2.3.2. Drug Loading and Encapsulation Efficiency. To determine doxorubicin loading in micelles, freeze-dried micelle samples were dissolved in dimethyl sulfoxide (DMSO), and drug concentrations were determined with a fluorescence spectrometer (Em: 485 nm, Em: 590 nm). Various concentrations of doxorubicin dissolved in DMSO (0.39, 0.78, 1.56, 3.125, and 6.25 μg/mL) were used to prepare the standard curve of doxorubicin based on their fluorescent intensities. To determine lapatinib loading in micelles, freeze-dried samples were dissolved with methanol and drug concentrations were determined with a HPLC method (Agilent Technologies 1200 series HPLC; DIKMA Suprisl-C 18 column; temperature 25 °C; mobile phase: methanol/H2O 85:15, 1 mL/min). Various concentrations of lapatinib dissolved in methanol (3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) were used to prepare the standard curve. Drug loading and encapsulation efficiency were then calculated using the following equations, respectively:

\[
\text{drug loading}(\%) = \left( \frac{\text{amount of loaded drug}}{\text{amount of polymer}} \right) \times 100\%
\]

\[
\text{encapsulation efficiency}(\%) = \left( \frac{\text{amount of loaded drug}}{\text{amount of drug added}} \right) \times 100\%
\]

\[
\text{theoretical loading}(\%) = \left( \frac{\text{amount of drug added}}{\text{amount of polymer}} \right) \times 100\%
\]

2.3.3. Morphology and Particle Size. The particle size distribution of micelles was determined by dynamic light scattering (Malvern Nano ZS). The morphology of micelles was determined with a transmission electron microscope using an acceleration voltage of 100 kV. Micelle samples were loaded on a copper grid and stained with 0.2% (w/v) phosphotungstic acid solution before test using transmission electron microscopy (TEM).

2.4. In Vitro Cytotoxicity. MCF-7 breast cancer cells and drug resistant MCF-7/ADR breast cancer cells were used for the in vitro cytotoxicity studies. MCF-7 and MCF-7/ADR cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and Keygen Biotech. Co., Ltd. (Nanjing, China). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate at 37 °C in humidified environment of 5% CO2. Doxorubicin (1 μg/mL) was added into the medium for MCF-7/ADR cells to maintain the drug resistance property. Cells were seeded in 96-well plates at a density of 6000 cells per well one day before treatment. Then, cells were treated with different formulations for additional 48 h and followed by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay or the lactate dehydrogenase (LDH) release study. Multiple doxorubicin concentrations were tested in each group including 0, 0.5, 1, 2, 4, 8, and 12 μM, respectively. During the optimization process, lapatinib at a dose of 10 μM or 15 μM was found to be able to effectively sensitize drug resistant MCF-7/ADR cells to DOX treatment, while not showing significant cytotoxicity by lapatinib itself. Therefore, these two lapatinib concentrations were selected for combination therapy.

For MTT assay, cell culture medium was replaced with 200 μL of fresh medium at the end of treatment. Then, 20 μL of MTT (5 mg/mL) solution was added and incubated for 4 h. The medium was carefully removed, and 200 μL of DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured in a microplate reader (Thermo, USA) at a wavelength of 570 nm. The relative cell viability was calculated with the following equation:

\[
\text{cell viability}(\%) = \frac{\text{OD}_{\text{test}} - \text{OD}_{\text{DMSO}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{DMSO}}} \times 100\%
\]

For the LDH release study, cell culture supernatant was harvested at the end of treatment and was analyzed with a LDH release assay kit (Betoye, Shanghai, China). The released LDH were expressed as the percentage of LDH released in cells treated with 1% Triton X-100.

2.5. Cellular Uptake Studies. Fluorescence Microscopy. The ability of micelles to transport doxorubicin into cells was determined using a fluorescence inverted microscope (Olympus IX81, E: 488 nm, Em: 570 nm). Cells were seeded at a density of 1 × 104 cells/well and cultured for 24 h. After treating cells with different formulations for 4 h at 37 °C, media were removed, and PBS was added to rinse cells for three times before observation with the microscope.

Flow Cytometry. Cells were plated at a density of 1 × 105 cells/well into 6-well microplates and cultured for 24 h. Then, cells were incubated at 37 °C for 4 h with different test articles diluted with fresh culture medium. At the end of treatment, cells were washed with PBS and digested with 0.25% trypsin. Then, cells resuspended in PBS were analyzed with a flow cytometer (BD Biosciences FACS Calif, Germany). The doxorubicin inherent fluorescence was detected as FL-2 (486/585 nm).

2.6. In Vivo Anticancer Efficacy Studies. Animal experiments were performed in accordance with the protocol approved by Institutional Animal Care and Use Committee (IACUC) of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Female BALB/c-nu nude mice (4–5
weeks old, 18−22 g) were housed under specific pathogen-free conditions. Animals possessed continuous access to sterilized food pellets and distilled water in a 12 h light/dark cycle. All of the animals were in quarantine for a week before treatment.

Tumor models were created by subcutaneously injecting MCF-7/ADR cells (1 × 10⁶ cells/mouse) into the back of BALB/c-nu nude mice with a 25 gauge needle. When tumors reached approximately 100 mm³, mice were randomly assigned to different treatment groups (n = 3). Different treatment formulations were injected in the tail vain of these mice according the following groups: Group 1, PBS (control); Group 2, DOX solution (7.5 mg/kg/3 d); Group 3, DOX micelles (7.5 mg/kg/3 d); Group 4, DOX micelles (7.5 mg/kg/3 d) + LAPA micelles (7.5 mg/kg/3 d). The tumor volume was measured and calculated using the following formula:

\[ V = \frac{W \times L}{2} \]

where W and L are the shortest and longest diameters, respectively.

The body weight of mice was also monitored during the course of the study. At day 21, mice were killed, and tumors were immediately harvested and weighed. In addition, major organs including heart, spleen, and kidney were harvested and subjected to a histological examination after H&E staining.

3. RESULTS

3.1. Synthesis and Characterization of Polymers. PEG-PBC copolymer was synthesized using DBU as a catalyst which gives a high conversion rate of monomer. The PEG-PBC polymer chemical structure was confirmed by ¹H NMR spectrum (Figure 1A), with the following characteristic peaks: δ 1.2 (CH₃ in BC unit), δ 3.6 (CH₂ in PEG), δ 4.3 (CH₂ in BC main chain), δ 5.2 (CH₂ in BC side group), and δ 7.3 (phenyl ring). All signals are assigned to methoxy poly(ethylene glycol) (mPEG) and polymerized BC units. The molecular weight of the PBC block of the polymer was 7000, as estimated based on the peak areas of PEG CH₂ groups at δ 3.6 and those of CH₂ in BC main chain at δ 4.3. We also characterized PEG-PBC polymer with GPC, which indicated a narrow molecular weight distribution with a PDI of 1.39 (Figure 1B). The molecular weights determined by GPC were \( M_m \) 10812, \( M_w \) 8686, and \( M_n \) 6268, respectively. The low CMC value (1.5 × 10⁻³ g/L) indicated the good stability of PEG-PBC micelles (Figure 1C).

3.2. Preparation and Characterization of Micelles. 3.2.1. Drug Loading Efficiency. To determine the ability of PEG-PBC to load doxorubicin (DOX) and lapatinib (LAPA), the loading efficiencies of these two drugs were calculated using the equations described in the experimental methods. For lapatinib, when we increase the theoretical loading from 5% to 25% (w/w), the drug solubility increased accordingly from 0.5 to 2.1 mg/mL, with loading efficiency decreased slightly from 98% to 84%. The solubility of lapatinib increased from 1.9 to 5.1 mg/mL, when the polymer concentration was increased from 5 to 25 mg/mL (Figure 2). Similar studies were also performed for doxorubicin loaded PEG-PBC micelles. Doxorubicin solubility increased from 0.3 to 0.85 mg/mL when the theoretical loading increased from 3% to 10%, with drug loading efficiency decreased from 97.4% to 94.8%. When polymer concentration increased from 10 to 30 mg/mL, the concentration of doxorubicin was increased accordingly from 0.7 to 2.1 mg/mL with a loading efficiency above 95% in all groups (Figure 3).

3.2.2. Morphology and Particle Size. The mean particle size of various micelle formulations determined with dynamic light
scattering (DLS) was around 100 nm, and there was little change in particle size due to the drug loading (data not shown). The particle size and morphology of these micelles were also determined using a transmission electron microscopy (TEM); TEM results showed that these micelles were spherical and had a narrow size distribution with a mean number average diameter less than 50 nm (Figure 4), which was lower than the Z-average diameters obtained from DLS. This was consistent with the previous report that the smaller particle size was observed with TEM than with DLS. The loading of doxorubicin or lapatinib did not cause any significant change in morphology and particle size (Figure 4). In addition, we also measured the effects of lyophilization/reconstitution process on the change of particle sizes. Results demonstrated that the lyophilization/reconstitution process did not lead to significant change in particle size of blank micelles as well as drug-loaded micelles (data not shown).

3.3. In Vitro Cytotoxicity. The cytotoxicity of various drug formulations was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For MCF-7 cells, there was no significant difference in cytotoxicity among free doxorubicin, doxorubicin micelle, doxorubicin + lapatinib (10 μM) micelle, and doxorubicin + lapatinib (15 μM) micelle (Figure 5A). The IC₅₀ was 1.2 μM doxorubicin for all four tested groups. The results indicated no enhanced internalization effect and no cytotoxic effect of lapatinib on cells under the test concentration. MCF-7/ADR cells displayed strong
resistance to free doxorubicin, leaving it virtually ineffective even with a high dose up to 12 μM. However, PEG-PBC micelles formulated doxorubicin exhibited improved activity compared to free doxorubicin, indicating its ability to overcome MDR. Notably, micelle-formulated doxorubicin plus 10 μM or 15 μM of lapatinib combination greatly increased the cytotoxicity. IC_{50} was 2 μM doxorubicin for these two combination therapy groups. The anticancer efficacy was comparable to that of sensitive MCF-7 cells, indicating the ability of lapatinib to sensitize drug resistant MCF-7/ADR cells and reverse drug resistance. Since lapatinib alone at 10 μM or 15 μM had no cytotoxicity on MCF-7/ADR cells, the observed anticancer efficacy in combination therapy might be attributed to the inhibition of MDR transporters rather than the inhibition of HER-2 or EGFR kinases (Figure 5B). There was no significant difference between the two LAPA + DOX groups with LAPA concentrations of 10 and 15 μM. No significant cytotoxicity was observed at PEG-PBC polymer concentration up to 1 mg/mL on both MCF-7 and MCF-7/ADR cells, suggesting its good biocompatibility (Figure 5C).

Furthermore, LDH release study was used to verify the in vitro anticancer activities. The results correlated well with the MTT assay data. In MCF-7 cells, the treatment of doxorubicin induced a concentration-dependent increase of LDH release, displaying no significant difference among different groups (Figure 6A). As expected, the free doxorubicin induced LDH release was significantly deceased in MCF-7/ADR cells. Doxorubicin micelles also showed a higher level of LDH release than free drugs. The combination of doxorubicin and lapatinib micelles showed highest activity among these three groups. No significant difference was observed between two combination therapy groups with different lapatinib concentrations (10 μM vs 15 μM, Figure 6B), indicating 10 μM was a sufficient dose for overcoming MDR. These in vitro cytotoxicity results revealed that micelle formulated doxorubicin and lapatinib when used as a combination therapy can effectively overcome MDR in breast cancer cells.

3.4. In Vitro Cellular Uptake Studies. To elucidate the mechanism that doxorubicin and lapatinib loaded micelles overcomes MDR, the cellular uptake of doxorubicin by MCF-7 and MCF-7/ADR was determined. Cells were treated with free doxorubicin, doxorubicin micelle (8 μM), and doxorubicin (8 μM) + lapatinib (10 μM) micelles. In MCF-7 cells, high levels of cellular uptake of doxorubicin were achieved in all of the three treatment groups with 91.8% positive cells in free doxorubicin group, 99.3% in doxorubicin micelle group, and 99.8% in doxorubicin (8 μM) + lapatinib (10 μM) micelle group, respectively (Figure 7A,B). In MCF-7/ADR cells, due to the overexpression of Pgp, almost no intracellular accumulation of doxorubicin was observed in the free doxorubicin treated group (1.6% positive cells). The micelle formulation significantly enhanced intracellular accumulation of doxorubicin as demonstrated by higher percentage of positive cells than that of free drug treated group. The addition of lapatinib (10 μM) further increased the percentage of positive cells in MCF-7/ADR cells (Figure 7A,B).

3.5. In Vivo Anticancer Studies. After characterizing the anticancer activities of the lapatinib and doxorubicin micelle combination therapy with in vitro studies, we moved forward to test their anticancer efficacies with an in vivo animal tumor model. Drug-resistant MCF-7/ADR cells were used to establish the tumor model. Though inhibition of tumor growth was achieved by both doxorubicin free drug and micelle formulations compared to the PBS control group, combination therapy with doxorubicin and lapatinib micelles showed the most potent anticancer efficacy. The inhibition of tumor growth was significantly enhanced in combination therapy group in comparison to the doxorubicin monotherapy groups. The tumor growth in the combination therapy group was completely arrested (Figure 8A). Tumor samples were collected at the end of study and weighted (Figure 8B,C). All of the groups that received chemotherapy agent showed significantly less tumor weight than the PBS control group. The tumor weight was the smallest in the doxorubicin + lapatinib micelle combination therapy group, which was significantly less than those in doxorubicin monotherapy groups. The change in body weight was also measured for preliminarily evaluation of the in vivo safety and tolerance (Figure 8D). The treatment with doxorubicin free drug showed most severe toxicity as indicated by the significant loss of body weight during the course of study. By contrast, neither micelle formulated doxorubicin nor doxorubicin plus lapatinib combination induced body weight loss. Moreover, the histological examination was found that the micelles did not cause obvious changes in the major organs (spleen, kidney, and heart), whereas the free DOX induced severe damage in spleen and kidney (Figure 9), suggesting the reduced toxicity and better tolerance of the micelle-based combination therapy.

4. DISCUSSION

Due to the significance of MDR in cancer therapy, many efforts have been made in developing different strategies to reverse MDR in cancers, which include the use of MDR transporter inhibitors as the adjuvants to sensitize resistant cancer cells to chemotherapy,\textsuperscript{18,38} the development of anticancer drugs which are not substrates for known MDR transporters,\textsuperscript{19,24} and the
inhibition of MDR transporters with gene silencing approach. The use of MDR transporter inhibitors to reverse MDR in cancer therapy has attracted great interest. Pgp are widely distributed in normal human tissues and cells, and they are responsible for protection of vital tissue or cells (e.g., central nervous system, bone marrow stem cells). The inhibition of Pgp will result in high chemotherapeutic agent concentration in these vulnerable tissues or cells and cause toxicities. Therefore, many treatment protocols using MDR inhibitors, though delayed the growth of tumors, failed to improve the overall survival rate of patients due to the severe toxicities.

In the current study, a combination therapy of lapatinib and doxorubicin was developed for the treatment of MDR breast cancers. Our results demonstrated that lapatinib significantly increased anticancer activities of doxorubicin on MCF-7/ADR MDR breast cancer cells. In contrast, lapatinib did not change the cytotoxicity of doxorubicin on MCF-7 cells (Figure 5). The treatment with lapatinib alone showed no effects on both MCF-7 and MCF-7/ADR cells at the tested concentrations. This observation is consistent with our previous studies where nontoxic concentrations of lapatinib enhanced cytotoxicity of paclitaxel on MDR prostate cancer cells. Several other studies have also identified lapatinib as an inhibitor for MDR transporters. Lapatinib as well as many other tyrosine kinase inhibitors (e.g., nilotinib, apatinib, and erlotinib) are modulators of ATP-binding cassette MDR transporters. Lapatinib can inhibit both Pgp and BCRP by direct binding to their ATP-binding site and minimize the efflux of chemotherapeutic agents which are the substrates of MDR transporters. Due to the inhibitory activity of lapatinib on Pgp, lapatinib can enhance the intracellular accumulation of doxorubicin in MDR cancer cells and thus reverse drug resistance and enhance cytotoxicity. To further confirm the mechanism that lapatinib overcomes MDR, we determined the effect of lapatinib treatment on the intracellular accumulation of doxorubicin in drug-resistant MCF-7/ADR cells. The intracellular accumulation of doxorubicin was determined with the fluorescence microscopy and the flow cytometry (Figure 7). A low level of intracellular accumulation of doxorubicin was observed in MCF-7/ADR cells when cells were treated with free doxorubicin alone. The low intracellular drug concentration was a major reason that MCF-7/ADR cells were

Figure 7. Cellular uptake of DOX on MCF-7 and MCF-7/ADR cells. (A) Fluorescence microscopy; (B) flow cytometry analysis of MCF-7 cells treated with free DOX (8 μM); DOX micelles (8 μM); DOX micelles (8 μM) + LAPA micelles (10 μM); and (C) flow cytometry analysis of MCF-7/ADR cells treated with free DOX (8 μM); DOX micelles (8 μM); DOX micelles (8 μM) + LAPA micelles (10 μM).
Figure 8. *In vivo* anticancer efficacy study. (A) Change of tumor sizes in MCF-7/ADR tumor-bearing nude mice received intravenous injection of different formulations; *a* *P* < 0.01, *b* *P* < 0.05, compared with the PBS control; + *P* < 0.05, compared with DOX monotherapy (free drugs or micelle formulations); (B) tumor size and (C) tumor weight measured at the end of study (21 days post the initiation of treatment). **a** *P* < 0.01, compared with the PBS control; ++ *P* < 0.01, compared with DOX + LAPA micelle combination. (D) Change of body weight in MCF-7/ADR tumor-bearing mice during the course of study. **a** *P* < 0.01, *b* *P* < 0.05, compared with the PBS control; # *P* < 0.05, compared with DOX micelles; + *P* < 0.05, compared with DOX + LAPA micelle combination. Points are presented as mean ± SD (n = 3).

resistant to doxorubicin monotherapy (Figures 5 and 7). The use of lapatinib significantly increased the intracellular accumulation of doxorubicin in MCF-7/ADR cells. This is consistent with our previous studies, which showed that lapatinib inhibited Pgp mediated drug efflux but had no effect on Pgp mRNA or protein levels. In summary, lapatinib overcomes MDR by directly inhibiting the activities of MDR transporters, thus reducing drug efflux and increasing the intracellular accumulation of doxorubicin in resistant cancer cells.

Currently, lapatinib has been used in combination therapy for treating HER-2 positive breast cancer cells. Many combination therapy protocols have been developed with lapatinib plus other therapeutic agents to treat refractory breast cancers. Lapatinib and other chemotherapeutic agents work on different mechanisms or targets which may enhance the anticancer activities through either synergistic or additive effects. The overexpression of EGFR and HER-2 are associated with a higher risk of breast cancer recurrence and with poor clinical outcomes. Lapatinib can inhibit these receptor mediated breast cancer growth and significantly improve the clinical outcomes. It was noted that the combination of lapatinib and doxorubicin in our study did not further enhance the cytotoxicity in nonresistant MCF-7 cells. The use of lapatinib alone did not have any anticancer effects on nonresistant MCF-7 or resistant MCF-7/ADR cells. These results are expected because MCF-7 cells are HER-2 negative breast cancer cells. However, we expect to have additional anticancer effects in HER-2 positive breast cancers by using lapatinib as an adjuvant in chemotherapy. Lapatinib will enhance the anticancer efficacy through two distinctive mechanisms: (1) inhibit HER-2 receptor mediated tumor growth; and (2) reverse MDR.
transporter mediated drug resistance. Due to the high prevalence of MDR and HER-2 overexpression in advanced breast cancers as well as their association with the aggressive cancer phenotype, the use of lapatinib as an adjuvant in chemotherapy will be a promising approach for treating refractory breast cancers.

Doxorubicin was selected in this study as a chemotherapeutic agent in combination with lapatinib. Doxorubicin is a first line drug for monotherapy or combination therapy to treat breast cancers in the clinics. The clinical use of doxorubicin is associated with toxic side effects. The situation becomes worse in patients with resistant breast cancers because a higher dose is needed to control the tumor growth, which may lead to more severe toxicities and side effects. A recent research reported that lapatinib strongly potentiated the cardiac toxicity of doxorubicin due to the inhibition of doxorubicin efflux in myocytes.49 Therefore, it is important to specifically deliver anticancer drugs and MDR inhibitors into tumor and minimize their exposure in normal tissues or organs. Nanocarriers can efficiently deliver drugs into tumors and thus have a great potential to be used as delivery systems for MDR inhibitor and chemotherapy drug combination therapy. In this study, PEG-PBC polymeric micelles were used for the delivery of lapatinib and doxorubicin. This polymer formed a stable micelle with a low critical micelle concentration (Figure 1). The stability of micelles is critical for drug retention and in vivo drug delivery. The benzyl moieties located at the hydrophobic block of PEG-PBC polymer are believed to stabilize the micelles by enhancing hydrophobic interaction. The benzyl ring may also interact with doxorubicin through $\pi-\pi$ interaction which will stabilize micelle as well as protect the encapsulated drugs from degradation.50 The prepared micelle formulations had a mean particle size of 100 nm, which is suitable for enhancing drug delivery into tumors via passive tumor targeting. Doxorubicin micelle monotherapy and lapatinib plus doxorubicin micelle combination therapy were well-tolerated and did not show significant systemic toxicity (Figure 8). This result is consistent with previous clinical studies using pegylated liposomal formulation of doxorubicin (PLD) plus oral lapatinib. Patients received that treatment showed low toxicities and no treatment-related cardiac toxicity.51

We observed that doxorubicin loaded PEG-PBC micelles showed greater anticancer activities than free doxorubicin in drug resistant MCF-7/ADR cells (Figure 5). Our results also demonstrated that doxorubicin micelles induced more LDH release in MCF-7/ADR cells (Figure 6). The cellular uptake studies showed that MCF-7/ADR cells treated with doxorubicin micelles had a higher intracellular drug concentration than cells treated with doxorubicin free drugs (Figure 7), which might be a reason that PEG-PBC micelles enhanced anticancer effects of doxorubicin in MCF-7/ADR cells. The enhanced intracellular accumulation of doxorubicin is possibly due to the inhibition of Pgp by the PEG-PBC polymer or due to the alteration of cellular uptake route.52 The PEG-PBC polymer used in our study is an amphiphilic molecule. Many amphiphilic molecules have been reported to be able to sensitize resistant cancer cells to chemotherapy. These amphiphilic molecules (e.g., pluronic, polyoxyl 15 hydroxy stearate <solutol HS15>, polyoxymethylene 20 stearyl ether <Brij 78>, and vitamin E TPGS) can deplete intracellular ATPs and inhibit MDR drug efflux transporters.53-56 These molecules may induce the change of mitochondrial membrane permeability, cause the loss of the membrane polarity, and lead to the depletion of ATPs. The enhanced drug accumulation and reversal of MDR may also due to the alteration in the route of cellular uptake and the change of subcellular localization of drugs encapsulated in micelles. Free drugs enter cells through simple diffusion and are sensitive to the effects of drug efflux transporters.

![Figure 9. Histological examination of heart, spleen, and kidney after treatment. The micelles caused little changes in the organs, but the free DOX induced severe toxicity (shrinking white pulp in the spleen and renal tubular necrosis).](image-url)
micelle encapsulated drugs enter cells through endocytosis which transports drugs into perinuclear regions where drugs are less susceptible to the effects of efflux transporters which are located in the cell membranes.32 This hypothesis has been investigated in several research papers, which have demonstrated the abilities of nanocarriers to reverse MDR in cancers.57–59

PEG-PBC micelles could efficiently load both lapatinib and doxorubicin. The solubility of these two drugs was significantly improved when PEG-PBC micelles were used to formulate them. Lapatinib showed higher drug loading in PEG-PBC micelles than doxorubicin (Figures 3 and 4). This was due to the difference in the chemical structures of lapatinib and doxorubicin, which leads to different compatibility of these two drugs with PEG-PBC polymers. The compatibility between a drug and a polymer (i.e., the miscibility or interaction between a drug and a polymer) is a key factor that determines the drug loading efficiency, stability, and drug release. Our previous studies have demonstrated the influence of polymer/drug compatibility on drug loading efficiency and release profiles.20,35 The drug/polymer compatibility and its relevance with drug loading could be studied by various methods including Flory–Huggins interaction parameters and molecular dynamics.36–62 Because of the difference in drug chemical structures, it is almost impossible to design a single polymer as a universal delivery carrier which will have high drug loading for all drugs. For combination therapy, the polymer used to formulate multiple drugs will not have the identical loading efficiency for different drugs. However, the selected polymer should have satisfactory loading efficiency for all the drugs in the combination therapy.

In conclusion, PEG-PBC polymeric micelle formulations have been developed for the delivery of lapatinib and doxorubicin as a combination therapy to treat resistant breast cancers. The use of lapatinib as an adjuvant significantly increased anticancer effects of doxorubicin in resistant breast cancer cells. The reversal of MDR was mainly due to the inhibition of MDR transporters by lapatinib. The combination therapy formulation was also evaluated with an in vivo animal tumor model and showed an inhibition of resistant breast cancer cells as well as a good tolerance in animals. These novel formulations have a great potential for treating resistant breast cancers.

REFERENCES


