Co-delivery of doxorubicin and P-gp inhibitor by a reduction-sensitive liposome to overcome multidrug resistance, enhance anti-tumor efficiency and reduce toxicity

Jie Tang1,2, Li Zhang1, Huile Gao1, Yayuan Liu1, Qianyu Zhang1, Rui Ran1, Zhirong Zhang1, and Qin He1

1Key Laboratory of Drug Targeting and Drug Delivery Systems, Ministry of Education, West China School of Pharmacy, Sichuan University, Chengdu, China and 2Department of Pharmaceutical Engineering, School of Bioengineering, Xihua University, Chengdu, China

Abstract

To overcome multidrug resistance (MDR) in cancer chemotherapy with high efficiency and safety, a reduction-sensitive liposome (CL-R8-LP), which was co-modified with reduction-sensitive cleavable PEG and octaarginine (R8) to increase the tumor accumulation, cellular uptake and lysosome escape, was applied to co-encapsulate doxorubicin (DOX) and a P-glycoprotein (P-gp) inhibitor of verapamil (VER) in this study. The encapsulation efficiency (EE) of DOX and VER in the binary-drug loaded CL-R8-LP (DOX + VER) was about 95 and 70% (w/w), respectively. The uptake efficiencies, the cytotoxicity, and the apoptosis and necrosis-inducing efficiency of CL-R8-LP (DOX + VER) were much higher than those of DOX and the other control liposomes in MCF-7/ADR cells or tumor spheroids. Besides, CL-R8-LP (DOX + VER) was proven to be uptaken into MCF-7/ADR cells by clathrin-mediated and macropinocytosis-mediated endocytosis, followed by efficient lysosomal escape. In vivo, CL-R8-LP (DOX + VER) effectively inhibited the growth of MCF-7/ADR tumor and reduce the toxicity of DOX and VER, which could be ascribed to increased accumulation of drugs in drug-resistant tumor cells and reduced distribution in normal tissues. In summary, the co-delivery of chemotherapeutics and P-gp inhibitors by our reduction-sensitive liposome was a promising approach to overcome MDR, improve anti-tumor effect and reduce the toxicity of chemotherapy.

Keywords

Co-delivery, liposomes, multidrug resistance, P-glycoprotein, reduction-sensitive

Introduction

Chemotherapy is the most common anticancer therapy (Bergh et al., 2001; Ragnhammar et al., 2001). However, its efficacy remains unsatisfactory due to two major factors: multidrug resistance (MDR) and toxicity (Rivera, 2010; Shi et al., 2011). Over-expression of the drug efflux transporter P-glycoprotein (P-gp) in the plasma membrane is a key mechanism responsible for MDR (Duhem et al., 1996; Dean & Allikmets, 2001), which leads to the reduced accumulation of anticancer drug inside cancer cells. Thus, higher doses are required which potentially increased the side effects of the therapeutics.

Nowadays, many strategies have emerged to overcome the P-gp barrier of chemotherapy, among which co-administrating P-gp inhibitors that possess “P-gp inhibition effect” represents the most common method. Whereas, P-gp is expressed not only in tumors but also in normal tissues, such as kidney, liver, adrenal glands, blood brain barrier and gastrointestinal track (Thiebaut et al., 1987; Patel et al., 2011). The non-selective binding of P-gp inhibitors with normal tissues blocks the efflux of anticancer drugs from normal tissues that may result in undesirable pharmacokinetic profiles and toxicity (Borowski et al., 2005; Wu et al., 2007). As an alternative approach, nanoparticles have drawn attentions as drug delivery systems to bypass P-gp on the surface of tumor cell membrane, i.e. “P-gp bypassing effect” (Wong et al., 2006; Kang et al., 2010). Note that the precondition for “P-gp bypassing effect” is the nanoparticles can be uptaken by cells, and the more efficient the cellular uptake is, the more obvious “P-gp bypassing effect” will be. However, amounts of P-gp also localized in the intracellular membranes, intracytoplasmic vesicles (Gervasoni et al., 1991), Golgi apparatus (Molinari et al., 1994), nucleus envelope (Calcabrini et al., 2000; Babakhanian et al., 2007), which makes it difficult for the released therapeutics in the cytoplasm to reach their intracellular targets and exert the therapeutic efficacy. Besides, the released drug in MDR cells could be extruded out again from cytoplasm to the outside of the plasma membrane. Therefore, co-encapsulation of cytotoxic drugs and P-gp inhibitors by the tumor-targeted carriers has become a good option for the treatment of MDR cancers (Jabr-Milane et al., 2008; Milane et al., 2011; Wang et al., 2012). The co-encapsulated system takes advantage of “P-gp bypassing effect” of nanoparticles and “P-gp inhibition effect” of P-gp inhibitors simultaneously (Figure 1), which
Chemotherapeutics was easily pumped out by P-gp overexpressed on MDR cells (route 1). Chemotherapeutics loaded nanoparticles could enter MDR cells via endocytosis and escape the P-gp efficiently by “P-gp bypassing effect”, while the released drug can be pumped out again by P-gp located on the cell membrane or on the organelle membrane (route 2). Chemotherapeutics and P-gp inhibitors co-loaded nanoparticles could increase drug accumulations in the cytoplasm and target organelles under the synergy of “P-gp bypassing effect” of nanoparticles and “P-gp inhibition effect” of P-gp inhibitors (route 3).

Figure 1. The intracellular delivery routes of different drug formulations. After arrival at the tumor site, the longer PEG_{5000} could be cleaved by the exogenous reducing agents, and R8 was thus exposed to mediate the internalization and lysosome escape efficiently (Wender et al., 2008; Futaki et al., 2013).

In this study, we applied this system to co-deliver anticancer drugs doxorubicin (DOX) and P-gp inhibitor verapamil (VER) for the targeted therapy of multidrug-resistant cancer (Figure 2A). Both DOX and VER were shown to display severe cardiotoxicity (Pennock et al., 1991; Bagalkot et al., 2006), which greatly limited their applications in vivo. Whereas in this system, DOX and VER were co-encapsulated in the liposomal system and simultaneously delivered to the tumor cells with high efficiency of tumor accumulation, cellular uptake and lysosomal escape, resulting in a reduced toxicity and synergistic antitumor effect. Furthermore, the main acting site of DOX is nucleus (Minotti et al., 2004; Coley, 2008), after being delivered into cells, intracellular VER could inhibit the nuclear P-gp to ensure DOX to reach its action target, and kill cancer cells through the induction of apoptosis and necrosis (Minotti et al., 2004; Coley, 2008). To verify the potential of this drug delivery system to reverse the MDR, its physicochemical properties were characterized first. Then the in vitro uptake efficiency, cytotoxicity, apoptosis and necrosis-inducing effect were performed on MCF-7 and MCF-7/ADR cells or tumor spheroids. The anticancer efficacy and safety in vivo was evaluated in MCF-7/ADR tumor bearing mice.

Materials and methods

Materials

Doxorubicin was a gift from Haizheng Pharmaceutical Co., Ltd. (Taizhou, China), and VER were purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, China). Soybean phosphatidylcholine (SPC) was purchased from Shanghai Taiwei Chemical Company (Shanghai, China). Cholesterol (CHO), cysteine (Cys) and MTT were purchased from Chengdu Kelong Chemical Company (Chengdu, China). The cholesterol anchored reduction sensitive PEG (CHO-S-S-PEG_{5000}) R8 modified PEG (CHO-PEG_{2000}-R8) and non-cleavable

PEG2000 was shielded by the longer cleavable PEG 5000. May further reduce the side effects of chemotherapeutics and P-gp inhibitors on normal tissues (Kaur et al., 2014). Currently, the applied tumor targeted carriers are far from perfect. One of the most essential reasons is that the widely used PEGylation strategy significantly hinders the carriers from entering into tumor cells (Tsukiioka et al., 2002), though it can help the carriers escape from the surveillance of reticuloendothelial system (RES) and enhance their accumulation in tumor tissues by the enhanced permeability and retention (EPR) effect (Zhang et al., 2014). Luckily, PEGylation with cleavable PEG has emerged and become an effective alternative to inherit the advantage of conventional PEG but does not affect the interactions between carriers and cell surface after the cleavage of PEG (Sun et al., 2013). Among various kinds of cleavable PEG, the cleavage of reduction-sensitive PEG based on disulfide linkage can be achieved by providing the exogenous reducing agent (Kuai et al., 2011), which is more controllable and universal compared to pH-sensitive and enzyme-sensitive cleavable PEG that rely heavily on the tumor microenvironment and the tumor type (Zhu & Torchilin, 2013). Another important reason to affect the treatment results of tumor targeted carriers is their limited ability of penetrating into cells (Torchilin, 2013). For many years, octaarginines (R8) belonging to positively charged cell penetrating peptides (CPPs) is applied to the surface decoration of carriers, which has already been accepted as an effective means to help carriers overcome plasma membrane barrier and increase their cellular uptake efficiently (Futaki et al., 2001). However, R8 lacks specificity and can penetrate any cells without selectivity, which limits its use in systemic administration (Maeda & Fujimoto, 2006). In addition, the positive charge of R8 made the R8 modified carriers easy to aggregate and captured by reticuloendothelial system (RES) in vivo. In our previous work (Tang et al., 2014), a multifunctional liposome (CL-R8-LP) co-modified with reduction-sensitive cleavable PEG and R8 has been successfully developed, which was proved to be an excellent tumor targeted drug delivery system with high efficiency of tumor accumulation and cellular uptake. During circulation, R8 that linked by a shorter PEG_{2000} was shielded by the longer cleavable PEG_{5000}.
PEG2000 (CHO-mPEG2000) were synthesized by our laboratory according to previously reported method (He et al., 2012). RPMI-1640 medium was purchased from GIBCO (Grand Island, NY). FITC Mouse Anti-Human P-glycoprotein (clone 17F9) antibody was purchased from BD PharMingen (San Diego, CA). Annexin V-FITC/PI apoptosis detection kit was purchased from KeyGEN Biotech (Nanjing, China). Lyso-tracker Blue DND-22 and agarose LMP was purchased from Invitrogen (Carlsbad, CA). Other chemicals and reagents were of analytical grade.

Preparation of liposomes

Blank liposomes were prepared by the lipid film hydration-ultrasound method (Qin et al., 2011), including reduction-sensitive co-modified liposomes (CL-R8-LP), made of SPC:CHO:CHO-PEG2000-R8:CHO-S-S-PEG5000 (65:26.2:0.8:8 molar% ratio), the control liposome (R8-LP), composed of SPC:CHO:CHO-PEG2000-R8 (65:34.2:0.8 molar% ratio) and the control liposome (CL-LP), made of SPC:CHO:CHO-S-S-PEG5000 (65:27:8 molar% ratio). All the lipids were dissolved in chloroform, then the organic solvent was removed by rotary evaporation at 37°C for 15 min. and the film was further dried in vacuum for 2 h.

To prepare different drug-loaded liposomes, the resulting lipid film was hydrated with (NH4)2SO4 solution (300 mM) at 37°C for 1 h, and further sonicated with a probe sonicator at 80 W for 150 s, then the external phase of liposomes was replaced with phosphate buffer saline (PBS) by passing through a Sephadex G50 (Pharmacia, Uppsala, Sweden) column (20 cm × 1.5 cm) that equilibrated with PBS (pH 7.4). DOX and VER in PBS was added to liposomes at a drug-to-lipid molar ratio of 1:10, respectively, and incubated at 45°C for 15 min. The total lipid concentration of liposomes was at 3 mM.

Characterization of liposomes

The particle size and zeta potential of liposomes were measured using Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK). The morphology of CL-R8-LP (DOX + VER) was observed under a transmission electron microscope (TEM) (JEM-100CX, JEOL, Tokyo, Japan). To determine the drug encapsulation efficiency (EE), the loaded drugs in liposomes and free drugs were first separated by a Sephadex G50 column (10 cm × 0.5 cm). The eluted fractions containing drug-loaded liposomes and free drugs were collected, respectively, and the drug-loaded liposomes were collected and lysed with equivoluminal methanol before determination. The amount of DOX and VER were determined by the high-performance liquid chromatography (HPLC) (Agilent, 1200, Santa Clara, CA) following experimental conditions: a Diamond C18 column (250 mm × 4.6 mm, pore size 5 µm), a mobile phase: 0.02 mol/L phosphate buffer (pH 3.5): acetonitrile (55:45, v/v), column temperature: 35°C, flow rate: 1.0 mL/min, and measured wavelength: 278 nm. The EE was calculated by the percent ratio of the weight of drug incorporated into liposomes to the initial total loading weight of drug. The in vitro release of DOX and VER from liposomes were investigated at 37°C in 50% FBS using a dialysis method (Wang et al., 2011). Briefly, 1 mL of drug-loaded liposome was mixed with the same volume of PBS containing 50% fetal bovine serum (FBS), sealed into dialysis tubes (molecular weight cutoff 14,000 Da), and immersed in 30 mL PBS (pH 7.4) at 37°C for 48 h with mild shaking. At predetermined time intervals, 2 mL of sample was withdrawn.

Figure 2. Characterization of liposomes. Schematic (A) and transmission electron microscopy images (B) of CL-R8-LP (DOX + VER). Release profiles of DOX (C) and VER (D) from different liposomes at 37°C in 50% FBS, data represent the mean ± SD (n = 3).
and replaced with an equal volume of fresh release medium. The collected samples were lyophilized and then dissolved in 0.2 mL of mobile phase and analyzed by HPLC under the same analytic conditions as described above. The accumulated release (%) was indicated by dividing the cumulative amount of drug recovered in the dialysis medium with the total amount in the liposomes.

**Cell lines and cell culture**

The human breast cancer MCF-7 cells and its multidrug resistant counterpart MCF-7/ADR cells were kindly donated from School of Pharmacy, Peking University (Beijing, China). The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 mM sodium azide, and maintained at 37°C in a humidified atmosphere containing 5% CO₂. MCF-7/ADR cells were exposed to 20 μM of CL-R8-LP (DOX + VER) for 0, 2, 4 h, respectively, followed by a treatment with the 50 nM of Lyso-tracker Blue DND-22 for 1 h to stain the lysosome. Then the cells were washed with cold PBS and observed with CLSM (Leica, Wetzlar, Germany).

**P-glycoprotein expression**

MCF-7 and MCF-7/ADR cells were allowed to grow in 6-well plates until 80% confluence. Cells were then collected, washed twice using BSA stain buffer (PBS containing 0.2% BSA and 0.09% NaCl) and resuspended in 50 μL BSA stain buffer to achieve a density of 2 × 10⁷ cells/mL. After that, 10 μL FITC Mouse Anti-Human P-glycoprotein were added and incubated with cells for 45 min at 4°C. After the incubation, cells were washed twice with BSA stain buffer and analyzed using flow cytometry (FACS) (Beckman Coulter, Fullerton, CA) for the green fluorescence intensity. Untreated cells were taken as controls for both cell lines.

**Cellular uptake study**

MCF-7 and MCF-7/ADR cells were seeded into 6-well plates at a density of 10⁵ cells per well and grown for 24 h, respectively. Different drug formulations were added to the plates, including DOX solution: DOX; DOX and VER solution: DOX + VER; liposomes only encapsulating DOX: CL-R8-LP (DOX); liposomes encapsulating both DOX and VER: R8-LP (DOX + VER), CL-R8-LP (DOX + VER) and CL-LP (DOX + VER). The final concentrations of DOX and VER (free or equivalent) used in the treatments were both 20 μM. After incubation in the absence Cys (−Cys), or in the presence of 10 mM Cys (+Cys) which was used to provide a reducing environment for the cleavage of reduction-sensitive PEG for 4 h at 37°C according to the previous studies (McNeeley et al., 2009; Kuai et al., 2010; Tang et al., 2014), the cells were harvested and analyzed by a confocal laser scanning microscope (CLSM) (Leica, Wetzlar, Germany) and FACS (Beckman Coulter, Fullerton, CA) according to the routine procedures (Kuai et al., 2010).

**Evaluation of uptake mechanism**

To explore the underlying uptake mechanism that was responsible for the internalization of CL-R8-LP (DOX + VER) in MCF-7/ADR cells, the uptake inhibition experiments were carried out. Cells were treated with 100 mM sodium azide, 800 μg/mL Poly-lysine, 5 μg/mL chlorpromazine, 5 μM nocodazole and 5 μg/mL filipin for 1 h at 37°C, respectively, prior to 4 h incubation with 20 μM of CL-R8-LP (DOX + VER) in the presence of Cys (10 mM). After that, cells were treated with cold PBS and analyzed by FACS (Beckman Coulter, Fullerton, CA). The samples received CL-R8-LP (DOX + VER) without the addition of inhibitors were used as control.

The co-localization of drug-loaded liposomes in lysosome was applied to investigate their intracellular trafficking pathway. The confluent cells were pre-incubated with 20 μM of CL-R8-LP (DOX + VER) for 0, 2, 4 h, respectively, and stained with Annexin V-FITC and PI following the manufacturer’s instructions. Fluorescence was measured by Annexin V-FITC/PI assay. MCF-7 and MCF-7/ADR cells were seeded into 96-well plates at a density of 2 × 10³ cells per well and grown for various concentration gradient of drug formulations. The final drug concentration gradient (free or equivalent) used in MCF-7 cells was 10, 5, 2.5, 1.25, 0.625, 0.3125 μM, respectively, while in MCF-7/ADR cells, the concentration gradient was 50, 20, 10, 5, 2.5, 1.25 μM, respectively. Following 12 h incubation at 37°C, the cells were washed with PBS and cultured in fresh medium for an additional 48 h. After treatment, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and the plate was incubated for 4 h at 37°C. The medium was replaced with 150 μL/well of dimethyl sulfoxide (DMSO) to dissolve purple formazan. The absorbance of the formazan solution at 570 nm, which is positively correlated to the cell viability, was measured by Varioskan Flash Multimode Reader (Thermo, Hudson, NH). Drug concentrations producing 50% cell growth inhibition was calculated and indicated as IC₅₀. Resistance index (RI) was calculated from dividing IC₅₀ of DOX by IC₅₀ of different DOX formulations in MCF-7 cells and that of different DOX formulations in MCF-7/ADR cells. Reversal efficiencies (RE) were calculated from dividing IC₅₀ of DOX by IC₅₀ of different DOX formulations in MCF-7/ADR cells.

**In vitro apoptosis assay**

Apoptosis and necrosis was quantified by Annexin V-FITC/PI analysis. MCF-7/ADR cells were seeded in 6-well plates at a density of 10⁵ cells per well and cultured overnight, followed by the incubation with different drug formulations at a drug concentration of 20 μM (free or equivalent) for 12 h. Then the cells were washed with PBS and cultured in fresh medium for an additional 48 h. After treatment, all cells were harvested and stained with Annexin V-FITC and PI according to the manufacturer’s instructions. Fluorescence was measured by FACS (Beckman Coulter, Fullerton, CA).

**Uptake and growth inhibition of tumor spheroids**

In order to produce the tumor spheroids of MCF-7 and MCF-7/ADR, 4 × 10³ cells in 0.1 mL of fresh medium was placed in a 96-well plate pre-coated with 2% agarose. After 5 days, spheroids of 200–300 μm in diameter were selected to use. Different drug formulations were added to the suspension
of spheroids to obtain a drug concentration of 20 μM and co-cultured for 4 h at 37 °C. The medium was then removed and the spheroids were rinsed with PBS before observing with CLSM (Leica, Wetzlar, Germany). For the growth inhibition assay, after incubating with different drug formulations for 12 h at 37 °C, the tumor spheroids were washed with PBS and cultured in fresh medium for 7 days. The largest (d_{max}) and smallest (d_{min}) diameters of each spheroid were determined using an inverted phase microscope fitted with an ocular micrometer, and spheroid volume was calculated using the following formula: \( V = \frac{\pi \times d_{max} \times d_{min}}{6} \). The inhibition ratio (\%) was calculated with the equation: Inhibition ratio (\%) = \( \frac{V_0 - V_i}{V_0} \times 100\% \), where \( V_i \) is the tumor spheroids volume at the i-th day after treatment, and \( V_0 \) is the spheroids volume prior to before treatment (Ballangrud et al., 1999; Xin et al., 2012).

MCF-7/ADR tumor bearing mice model

Female BALB/c nude mice weighing 20 ± 2 g were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China) and treated according to the protocols evaluated and approved by the Experimental Animals Administrative Committee of Sichuan University. The MCF-7/ADR tumor bearing mice model was established by subcutaneous inoculation of 2 × 10^6 cells in the right oxters.

In vivo anti-tumor efficacy

The MCF-7/ADR tumor bearing mice (the tumor volumes were approximately 50 mm^3) were randomly assigned to the following seven treatment groups (n = 6) and administrated with saline and different drug formulations (free or equivalent, the dose of DOX and VER was 2 mg/kg DOX and/or 2 mg/kg VER) via the vein every 4 day for five times. And each drug administration was followed by the Cys (120 mg/kg) or PBS injection 24 h later. The Cys administration time and dose were chosen according to the previous study (Tang et al., 2014), which could ensure the strongest accumulation of CL-R8-LP in tumor and the efficient cleavage of the cleavable PEG in vivo, respectively. Body weight and tumor volumes were recorded for every two days. Tumor volume prior to before treatment (Ballangrud et al., 1999; Xin et al., 2012).

In vivo safety evaluation

To study the safety of the liposomal drug delivery system, 20 MCF-7/ADR tumor bearing mice were randomly divided into 4 groups (n = 5) and intravenously injected with saline, DOX + VER, CL-R8-LP (DOX + VER) and drug-free CL-R8-LP co-administrating with Cys (+Cys). The administration dose and frequency were kept the same as described in the above paragraph. On day 18, the mice were sacrificed, blood samples were collected for hematological and biochemistry analysis. For the hematological assessment, white blood cell (WBC), red blood cell (RBC) and platelet (PLT) were counted by MEK-6318K Automatic Hematology Analyzer (Nihonkohden, Shinjuku-ku, Japan). And for the biochemistry analysis, alanine aminotransferase (ALT), aspartate transaminase (AST), creatine kinase (CK), lactate dehydrogenase (LDH), urea nitrogen (BUN) and creatinine (CREA) levels were measured by Hitachi 7020 automatic biochemical analyzer (Hitachi Ltd. Hyogo, Japan). Simultaneous, major organ tissue samples (heart, liver, spleen, lung and kidney) were obtained for histologic analysis. Their sections were processed for routine H&E staining, and then visualized under optical microscope.

Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed using Student’s t-test and ANOVA. The differences were considered significant when p < 0.05.

Results

Characterization of liposomes

All drug-loaded liposomes except R8-LP (DOX + VER) exhibited mean particle diameters smaller than 100 nm, good polydispersity (PDI < 0.2) and almost neutral zeta potentials (Table 1). The representative TEM of CL-R8-LP (DOX + VER) revealed a discrete and uniform spherical appearance and the size correlated well with the above results measured by zetasizer (Figure 2B). The encapsulation efficiency of DOX and VER in different liposomes were about 94 and 70% (w/w), respectively. The release profiles of drugs in CL-R8-LP and CL-LP was less than 20% after 24 h dialysis (Figure 2C and D), exhibiting a good sustained-release profile and stability. As the exposure of positive charge of R8 would increase the instability of liposomes (Sharma et al., 2013), the PDI and cumulative release of R8-LP was relatively higher than that of CL-LP in which no R8 included and higher than that of CL-R8-LP, which had a longer PEG aqueous layer providing shielding effect on R8.

P-gp expression

MCF-7 and MCF-7/ADR cells were evaluated for the expression of the P-gp on the cellular surface. MCF-7/ADR cells was observed with significantly higher expression of P-gp compared with MCF-7 cells (Figure 3A), which made this pair a good model to evaluate the MDR reverse effect.

Cellular uptake study

The cellular uptake of DOX, DOX + VER, CL-R8-LP (DOX)/(+Cys), CL-R8-LP (DOX + VER)/(+Cys) and R8-LP (DOX + VER) by MCF-7 cells exhibited no significant differences (Figure 3C), indicating that effective cellular uptake was achieved by both free drug and drug-loaded liposomes, and the addition of the P-gp inhibitor VER did not impact the cellular uptake efficiency in MCF-7 cells. However, in MCF-7/ADR cells, DOX was not effectively uptaken due to the P-gp expressed on the plasma membrane. The fluorescent intensity of DOX + VER was 2.90 times stronger than that of DOX, and CL-R8-LP (DOX + VER)/
(+Cys) was 1.50 times the fluorescent intensity of CL-R8-LP (DOX)/(+Cys), which indicated that the cellular uptake and intracellular accumulation of DOX in MCF-7/ADR cells could be significantly increased due to “P-gp inhibition effect” of VER. Besides, CL-R8-LP (DOX)/(+Cys) could also increase the cellular uptake by 0.56 times compared to DOX via “P-gp bypassing effect” of the co-modified liposome. Thus, by the synergy of “P-gp bypassing effect” and “P-gp inhibition effect”, CL-R8-LP (DOX + VER)/(+Cys) was 2.31 times the uptake efficiency of DOX.

Table 1. The size, PDI, Zeta potential and encapsulation efficiency/EE (%) of different liposomes.

| Liposomes                  | Size (nm) ± SD  | PDI ± SD  | Zeta (mV) ± SD | DOX (%) ± SD  | VER (%) ± SD  |
|----------------------------|-----------------|-----------|----------------|---------------|---------------|
| CL-R8-LP (DOX)            | 90.2 ± 2.2      | 0.123 ± 0.013 | −0.03 ± 1.59   | 95.33 ± 1.58  | \             |
| CL-R8-LP (VER)            | 89.7 ± 3.0      | 0.112 ± 0.020 | 0.08 ± 0.62    | \             | 71.43 ± 1.10  |
| CL-R8-LP (DOX + VER)      | 91.3 ± 2.7      | 0.138 ± 0.017 | 0.14 ± 1.35    | 94.96 ± 1.32  | 70.48 ± 1.45  |
| R8-LP (DOX + VER)         | 125.4 ± 4.9     | 0.209 ± 0.024 | 5.60 ± 1.41    | 95.89 ± 0.79  | 72.93 ± 1.02  |
| CL-LP (DOX + VER)         | 90.5 ± 1.6      | 0.127 ± 0.016 | −0.58 ± 0.72   | 96.08 ± 0.81  | 71.98 ± 0.93  |

Data represent the mean ± SD (n = 3).
In addition, CL-LP (DOX + VER)/(+Cys) and CL-R8-LP (DOX + VER)/(-Cys) with no R8 attached or exposed, displayed significantly lower uptake efficiencies than those of CL-R8-LP (DOX + VER)/(+Cys) and R8-LP (DOX + VER) in both types of cells, which indicated that the prerequisite for ‘‘P-gp bypassing effect’’ and ‘‘P-gp inhibition effect’’ is the binary-drug-loaded liposomes, which could be efficiently uptaken by the cells. And the above results were consistent with the qualitative determination results (Figure 3B).

Evaluation of uptake mechanism

To investigate the cellular uptake mechanism of CL-R8-LP (DOX + VER)/(+Cys), MCF-7/ADR cells were pretreated with different endocytosis inhibitors before the addition of liposomes (Figure 4B). Treatments of sodium azide and polylysine (positive charge inhibitor) strongly inhibited the CL-R8-LP (DOX + VER) internalization to 36.7 and 23.8% of control, respectively, which suggested that the endocytosis of CL-R8-LP (DOX + VER) by MCF-7/ADR cells was energy- and charge-dependent. Clathrin-, caveolin- and macropinocytosis-mediated endocytosis are three main mechanism of endocytosis (Wang et al., 2010). Therefore, we investigated the cellular uptake of CL-R8-LP (DOX + VER) in the presence of chlorpromazine (an inhibitor for clathrin-mediated endocytosis), nocodazole (a specific inhibitor of macropinocytosis-mediated endocytosis) and filipin (an inhibitor for caveolin-mediated endocytosis). Filipin was shown to have no significant impact on the internalization of CL-R8-LP (DOX + VER), while chlorpromazine and nocodazole decreased the internalization to 59.4 and 46.5% of control, respectively, which indicated that the endocytosis of CL-R8-LP (DOX + VER) in the MCF-7/ADR cells was mediated via multiple pathways, including clathrin-mediated and macropinocytosis-mediated endocytosis.

The co-localization of DOX and lysosomes was employed to evaluate the intracellular trafficking pathway of CL-R8-LP (DOX + VER). As shown in Figure 4(A), the red fluorescence of liposomal DOX was hardly observed in lysosomes (blue), suggesting that DOX escaped from lysosomes efficiently with the increase of incubation time. Under synergistic effect of VER, DOX could enter and specifically distribute to nucleus with high efficiency (nucleus was delineated with the white dotted line in bright field).

MDR reversal by cytotoxicity assay

Cytotoxicity of various DOX formulations was determined in MCF-7 and MCF-7/ADR cells using an MTT assay. As shown in Table 2, IC_{50} values of the various formulations in MCF-7/ADR cells were in the following order: DOX + VER < CL-R8-LP (DOX + VER)/(+Cys) < R8-LP (DOX + VER) < CL-R8-LP (DOX)/(+Cys) < DOX (CL-

Figure 4. (A) The co-localization of DOX in lysosome after the MCF-7/ADR cells incubating with CL-R8-LP (DOX + VER)/(+Cys) for 0, 2, 4 h, respectively. (B) The endocytosis inhibition assay on MCF-7/ADR cells. The inhibition rate (%) is expressed as the ratios of the cellular uptake in the presence of various inhibitors to the uptake in the absence of inhibitor. Data represent the mean ± SD (n = 3). *p < 0.001, N.S.: No significant difference, versus control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
Table 2. The IC₅₀, the corresponding resistance index (RI) and Reversal efficiencies (RE) of different DOX formulations in MCF-7 and MCF-7/ADR cells.

| Drug formulations | IC₅₀ (µM) | MCF-7 | MCF-7/ADR | RIa | REb |
|-------------------|----------|-------|-----------|-----|-----|
| DOX + VER         | 1.24 ± 0.09 | 67.61 ± 6.50 | 54.53 ± 1.00 |  |    |
| DOX + CL-R8-LP (DOX)/(+Cys) | 1.22 ± 0.07 | 47.63 ± 3.96 | 39.07 ± 1.42 |  |    |
| DOX + CL-LP (DOX)/(+Cys) | 2.39 ± 0.22 | / | / |  |    |
| DOX + CL-R8-LP (DOX + VER)/(−Cys) | 0.91 ± 0.03 | 18.95 ± 1.79 | 20.90 ± 3.57 |  |    |
| DOX + R8-LP (DOX + VER) | 0.93 ± 0.02 | 20.75 ± 3.04 | 22.43 ± 3.26 |  |    |
| DOX + CL-LP (DOX + VER)/(+Cys) | 2.53 ± 0.11 | / | / |  |    |

Data represent the mean ± SD (n = 3).

RIa was calculated from the ratios between IC₅₀ of different DOX formulations in MCF-7/ADR cells and that of different DOX formulations in MCF-7 cells.

REb were calculated from dividing IC₅₀ of DOX by IC₅₀ of different DOX formulations in MCF-7/ADR cells.

R8-LP (DOX + VER)/(−Cys) ~ CL-LP (DOX + VER)/(+Cys). The IC₅₀ of DOX + VER was 4.54 times lower than that of DOX, and the IC₅₀ of CL-R8-LP (DOX + VER)/(+Cys) was 1.51 times lower than that of CL-R8-LP (DOX)/(+Cys), further demonstrating that under ‘‘P-gp inhibition effect’’ of VER, the intracellular accumulation of DOX in MDR cells increased, then producing stronger cytotoxicity. In addition, via ‘‘P-gp bypassing effect’’ of co-modified liposome, more DOX could be delivered into MDR cells by CL-R8-LP (DOX)/(+Cys), then decrease IC₅₀ value by 0.41 times compared to DOX. While the IC₅₀ values of CL-R8-LP (DOX + VER)/(−Cys) and CL-LP (DOX + VER)/(+Cys) were too high to determine, which indicated that a proper carrier that could enter cells with high efficiency was critical. Besides, the IC₅₀ of VER and CL-R8-LP (VER)/(+Cys) encapsulating VER alone in MCF-7 and MCF-7/ADR cells was too high and the cytotoxicity of VER at the used dosage could be neglected (Supplementary data; Figure 1). As a result, VER and CL-R8-LP (VER)/(+Cys) encapsulating VER were not set as control groups in our study, MCF-7/ADR cells exhibited strong resistance against DOX, and the resistance index (RI) of DOX was 54.43, which was much higher than some other DOX formulations, including CL-R8-LP (DOX)/(+Cys), R8-LP (DOX + VER), CL-R8-LP (DOX + VER)/(+Cys) and DOX + VER. Effects of different DOX formulations on MCF-7/ADR cells were further expressed in the form of reversal efficiencies (RE) directly (Kabanov et al., 2002), the results showed DOX + VER, CL-R8-LP (DOX + VER)/(+Cys), R8-LP (DOX + VER) had obvious MDR reversal effect, and the RE of CL-R8-LP (DOX + VER)/(+Cys) was 1.51 times higher than that of CL-R8-LP (DOX)/(+Cys).

In vitro apoptosis assay

Annexin V-FITC/PI staining assay was performed to further study the apoptosis and necrosis that was induced by different drug-loaded liposomes. As shown in Figure 5(A), the lower left quadrant (negative for both Annexin V and PI) showed normal viable cells. The lower right quadrant (only positive for Annexin V-FITC) was used for early apoptotic cells. Both the upper right (double-positive for Annexin V-FITC and PI) and upper left (only positive for PI) quadrants were used for late apoptotic/necrotic cells. In Figure 5(B), the apoptosis and necrosis rate (%) of cells represented the total percentage of early apoptotic cells and late apoptotic/necrotic cell. As a result, the DOX combined with VER treatment of MCF-7/ADR cells induced a higher apoptosis and necrosis rate compared with DOX alone, no matter whether it was in the form of free drug or liposome encapsulated drugs. The apoptosis and necrosis rate of cells treated with CL-R8-LP (DOX)/(+Cys) slightly increased by 0.66 times compared to DOX. CL-R8-LP (DOX + VER)/(+Cys) could induce a large amount of cells apoptosis and necrosis as more DOX were uptaken and accumulated in MDR cell via ‘‘P-gp inhibition effect’’ of VER and ‘‘P-gp bypassing effect’’ of CL-R8-LP. Additionally, the apoptosis and necrosis rate of cells treated with CL-R8-LP (DOX)/(+Cys) and R8-LP (DOX + VER) was significantly higher than that of CL-R8-LP (DOX + VER)/(−Cys) and CL-LP (DOX + VER)/(+Cys) treated cells, which implied the efficient cellular uptake of carriers was a prerequisite to the DOX-induced apoptosis and necrosis.

Uptake and growth inhibition of tumor spheroids

The cellular uptake and antitumor activity of DOX formulations were evaluated by using three-dimensional multicellular spheroids as the in vitro model, which closely represent the in vivo situation compared to traditional monolayer culture system (Minchinton & Tannock, 2006; Wang et al., 2013; Gao et al., 2014). Apparently, the strong fluorescence signal of DOX was observed in MCF-7 tumor spheroids that incubated with DOX, DOX + VER, CL-R8-LP (DOX)/(+Cys), CL-R8-LP (DOX + VER)/(+Cys) and R8-LP (DOX + VER) (Figure 6A). Moreover, the fluorescence intensity of these groups did not show significant differences. However, since MCF-7/ADR tumor spheroid model retained the resistance ability against DOX, much weaker fluorescence signal was observed in the MDR tumor spheroids from the DOX group, and the combination of DOX and VER remarkably enhanced the cellular uptake in tumor spheroids. The growth inhibition assay was used to evaluate the cytotoxicity of different drug formulations to tumor spheroids. As shown in Figure 6(B), all drug formulations except CL-R8-LP (DOX + VER)/(−Cys) and CL-LP (DOX + VER)/(+Cys) inhibited the growth of MCF-7 tumor spheroids, and the inhibition ratios were up to 50%. Whereas the inhibition ratio of MCF-7/ADR tumor spheroids treated with DOX remarkably decreased due to the resistance to DOX. The inhibition ratio of MCF-7/ADR tumor spheroid treated with CL-R8-LP (DOX + VER)/(+Cys) increased by 48.93% compared to DOX, and increased by 17.89% compared to CL-R8-LP (DOX)/(+Cys), indicating that CL-R8-LP (DOX + VER)/(+Cys) had a better MDR reversal ability.

In vivo anti-tumor efficacy

In vivo anticancer activity of CL-R8-LP (DOX + VER)/(+Cys) was performed in MCF-7/ADR tumor bearing mice. As shown in Figure 7(A), the tumor volumes of saline group increased dramatically over time, while tumor volume
Figure 5. The apoptosis assay on MCF-7/ADR cells after treatment with different drug formulations. (A) The representative quadrant plot obtained by FACS analysis showing the effect of drug formulations on initiation of apoptotic activity in vitro. (B) The proportion of apoptotic and necrotic MCF-7/ADR cell death (%) after different DOX formulation treatment. (a) Blank; (b) DOX; (c) DOX + VER; (d) CL-R8-LP (DOX)/(+Cys); (e) CL-R8-LP (DOX + VER)/(-Cys); (f) CL-R8-LP (DOX + VER)/(+Cys); (g) R8-LP (DOX + VER); (h) CL-LP (DOX + VER)/(+Cys). The concentration of DOX (free or equivalent) in the cell culture was 20 μM. Data represent the mean ± SD (n = 3). *p < 0.001, N.S.: No significant difference, versus CL-R8-LP (DOX + VER)/(+Cys) group.

Figure 6. (A) Representative CLSM images of MCF-7 and MCF-7/ADR tumor spheroids incubated with different drug formulations at 37 °C for 4 h. (B) Inhibition ratio (%) of tumor spheroids 7 days after treatment with different drug formulations at 37 °C. (a) Blank; (b) DOX; (c) DOX + VER; (d) CL-R8-LP (DOX)/(+Cys); (e) CL-R8-LP (DOX + VER)/(+Cys); (f) CL-R8-LP (DOX + VER)/(+Cys); (g) CL-R8-LP (DOX + VER)/(+Cys); (h) CL-LP (DOX + VER)/(+Cys), data represent the mean ± SD (n = 3). *p < 0.001, N.S.: No significant difference, versus corresponding CL-R8-LP (DOX + VER)/(+Cys) group.
remained unchanged in CL-R8-LP (DOX + VER)/(+Cys) treated group. In contrast, other treatment groups showed a relatively weaker inhibitory effect on the tumor growth. Eighteen days after the initiation of treatment, the tumors from each treatment group were obtained, photographed (Figure 7C) and weighted (Figure 7D). The tumor weight of CL-R8-LP (DOX + VER)/(+Cys) treated group was 2.85 and 1.17 times lighter than that of saline group and DOX + VER treated group, respectively. Furthermore, the tumor H&E staining (Figure 7E) demonstrated that CL-R8-LP (DOX + VER)/(+Cys) group prominently generated more advanced necrosis areas compared with other groups. Thus,
CL-R8-LP (DOX + VER)/(+Cys) were shown to provide a better antitumor efficiency in vivo. At the same time, as a reflection of the system toxicity, weight loss of mice was monitored throughout the treatment period (Figure 7B), the mice treated with saline or liposomal encapsulated drugs showed less body weight shift throughout the entire experiment, indicating better drug tolerability and low degree of systemic toxicity. In contrast, the body weight loss was remarkable in the DOX + VER group, which might be induced by the cardiotoxicity of DOX and VER.

**In vivo safety evaluation**

For safety purposes, the systematic toxicity of CL-R8-LP (DOX + VER)/(+Cys) was evaluated in MCF-7/ADR tumor-bearing mice. As shown in Table 3, the DOX + VER treated mice showed a significant decrease in WBC count compared to the saline group (p < 0.01), indicating a potential side effect to the immune system (Ishida et al., 2005). Biomarkers, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used as an indicator of liver injury (Ozer et al., 2010), and aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) have been widely used for the diagnosis of cardiac diseases (Goldberg & Winfield, 1972; Sobel & Shell, 1972), while BUN and CREA were used to predict the kidney function (Xin et al., 2012). As shown in Table 4, the content of ALT, AST, CK, LDH in DOX + VER treated mice was significantly higher than that of the saline control group, indicating severe hepatic or cardiac toxicity was induced by the free drugs within the dosage frame. For CL-R8-LP (DOX + VER)/(+Cys) and CL-R8-LP/(+Cys) treated groups, all biomarkers measured showed no significant differences compared with the saline control groups. Besides, no obvious renal toxicity was observed among all treatment groups. Furthermore, H&E stained sections of the main organs, including heart, liver, spleen, lung and kidney, were examined. For the CL-R8-LP (DOX + VER)/(+Cys) and CL-R8-LP/(+Cys) treated groups, all the major organs were normal, without obvious histopathological abnormalities or lesions, and were similar to the saline control group, indicating no cell or tissue damage. In contrast, serious cardiotoxicity was induced by DOX + VER as indicated by the appearance of hyperemia, myocardial fiber breakage and necrosis with acute inflammatory cell infiltration (Figure 8).

**Discussion**

The limited clinical outcomes and obvious toxicity of conventional chemotherapy in treating the MDR cancer promoted the development of new targeted drug delivery systems. All the drug delivery systems would face several barriers in vivo, the first one is the ‘kinetic barrier’, which means recognition and clearance by the RES (Frank, 1993; Tseng et al., 2009); the second barrier is the P-gp over-expressed plasma membrane (Duhem et al., 1996; Dean & Allikmets, 2001); in addition, the P-gp over-expressed membranes of target organelles is also a key factor to prevent the drug from working in MDR cancer cell (Gervasoni et al., 1991; Molinari et al., 1994; Calabrini et al., 2000; Babakhian et al., 2007). Then an ideal system should overcome the above barriers with high efficiency. Furthermore, it needs to possess excellent bio-safety because reducing the side effect of therapeutics is another great concern.

In this study, we developed a reduction-sensitive liposome co-encapsulating DOX and VER, and various experiments were performed to evaluate its efficiency of overcoming barriers. Since the amount of P-gp expressed on the membranes of MCF/ADR cells (Figure 3A), the uptake efficiency of DOX in MCF-7/ADR cells were much lower than that of in MCF-7 cells (Figure 3B and C), while CL-R8-LP (DOX + VER)/(+Cys) enhanced the uptake efficiency in MCF-7/ADR cells by 2.31 times compared to DOX via ‘P-gp bypassing effect’ of CL-R8-LP and ‘P-gp inhibition effect’ of VER. Besides, the cellular uptake of DOX + VER was higher than that of CL-R8-LP (DOX + VER)/(+Cys), which might be induced by different pathways into cells. Above results were consistent with those of tumor spheroids uptake (Figure 6A) and demonstrated that CL-R8-LP (DOX + VER)/(+Cys) could get through the plasma membrane barrier with high efficiency and achieve accumulation in the MDR cells.

The cellular uptake mechanism and intracellular trafficking of CL-R8-LP (DOX + VER)/(+Cys) were investigated to evaluate the intracellular fate of drugs. Previous researches suggested that the efficient lysosome escape of drugs was favorable in killing the MDR cells, the main reasons were as follows: (1) Drugs could avoid degradation and expiring in lysosomes (Thierry et al., 1993; Yang et al., 2012). (2) Lysosome, belonging to endomembrane system of eukaryotic

### Table 3. The blood cell levels in MCF-7/ADR-bearing mice after treatment (n = 5).

| Groups          | WBC (×10³/L) | RBC (×10¹²/L) | PLT (×10⁹/L) |
|-----------------|--------------|---------------|--------------|
| Saline          | 2.98 ± 0.47  | 8.32 ± 0.76   | 409 ± 55     |
| DOX + VER       | 1.63 ± 0.42a | 7.73 ± 0.36   | 388 ± 60     |
| CL-R8-LP (DOX + VER)/(+Cys) | 2.87 ± 0.21  | 7.84 ± 1.16   | 411 ± 37     |
| CL-R8-LP/(+Cys) | 2.82 ± 0.91  | 7.85 ± 0.52   | 406 ± 75     |

*p < 0.01, versus saline group.

### Table 4. The serum biomarkers in MCF-7/ADR-bearing mice after treatment (n = 5).

| Groups          | ALT (IU/L) | AST (IU/L) | CK (UL) | LDH (U/L) | BUN(mM) | CREA (µM) |
|-----------------|------------|------------|---------|-----------|---------|-----------|
| Saline          | 24.6 ± 5.5 | 68.4 ± 19.4| 469.2 ± 185.3| 573.0 ± 84.9| 5.8 ± 0.4| 18.4 ± 3.8|
| DOX + VER       | 31.2 ± 6.1a| 204.8 ± 56.6| 2802.7 ± 725.2a| 1299.3 ± 391.8b| 6.4 ± 1.1| 23.2 ± 4.5|
| CL-R8-LP (DOX + VER)/(+Cys) | 27.5 ± 7.3 | 84.5 ± 17.0 | 700.3 ± 171.5 | 623.3 ± 99.6 | 6.1 ± 1.0 | 21.3 ± 3.9 |
| CL-R8-LP/(+Cys) | 26.4 ± 4.7 | 82.4 ± 21.2 | 636.8 ± 207.7 | 522.0 ± 97.8 | 6.2 ± 0.9 | 17.4 ± 3.0 |

*p < 0.05, *p < 0.01, **p < 0.001, versus saline group.
cells, exchanged with plasma membrane via vesicular transport (Farquhar, 1983; Lippincott-Schwartz et al., 1991), which could be involved in the drug transport from the perinuclear region to the cell periphery, and increasing the effect of P-gp on the plasma membrane (Szaflarski et al., 2013). Thus, escape from lysosome could decrease the pump-out of intracellular drugs. (3) The free drugs or drug delivery systems that escaped from lysosome could quickly reach each target organelles (such as mitochondria, Golgi or nucleus), and played efficacy. The results in our study showed CL-R8-LP (DOX + VER)/(+Cys) enter MCF-7/ADR cells via clathrin-mediated and macropinocytosis-mediated endocytosis (Figure 4B). Macropinocytosis-mediated endocytosis was reported to facilitate the lysosome escape of delivery system (Wender et al., 2008), which was further validated by the result of lysosome co-localization assay (Figure 4A). After rapidly escaping from lysosome to cytoplasm, the encapsulated contents (DOX and VER) might be either released from CL-R8-LP, or retained in CL-R8-LP. Furthermore, the signal of DOX was clearly shown in cell nucleus (Figure 4A), indicating that under synergy of VER, the intracellular DOX could further overcome the barrier of P-gp over-expressed at the nuclear membrane and accumulated in nuclear efficiently. Since the main action target of DOX is nucleus and it kills cancer cells through the induction of apoptosis and necrosis in a dose-dependent way (Minotti et al., 2004; Coley, 2008), the more DOX accumulated in the nucleus, the stronger cytotoxicity, apoptosis and necrosis would be observed in MCF-7/ADR. As expected, CL-R8-LP (DOX + VER)/(+Cys) displayed the highest reversal cytotoxicity (Table 2), cell apoptosis and necrosis rate (Figure 5) and growth inhibition to tumor spheroids among all treatment groups except for (DOX + VER) group.

Although the in vitro cellular uptake and cytotoxicity of DOX + VER and R8-LP (DOX + VER) were significant, the anti-tumor effect was far from satisfactory due to a lack of specificity in vivo (Figure 7). In addition, as mentioned in the introduction, the positive charge of R8 would increase the instability of R8-LP in vivo, which further decreased the treatment efficacy of R8-LP (DOX + VER). In our previous study (Tang et al., 2014), the bio-distribution study showed that CL-R8-LP efficiently escaped from RES and accumulated in tumor tissues via the EPR effect owing to the “stealth” properties of the reduction-sensitive cleavable PEG.
and its masking effect to the non-specificity of R8. In the present study, the positive charge (Table 1) and the in vitro uptake efficiency of CL-R8-LP (DOX + VER)/(+Cys) by MCF-7/ADR cells and tumor spheroids (shown in Figure 3B and C; Figure 6A) were much lower than that of R8-LP (DOX + VER), which also verified the shielding effect of cleavable PEG on R8 in the absence of Cys. Thus, CL-R8-LP could render drugs with the passive tumor target ability, then resulted in an increase of in vivo anti-tumor efficacy in CL-R8-LP (DOX + VER) group compared to R8-LP (DOX + VER) and DOX + VER groups. Hence, CL-R8-LP (DOX + VER) was demonstrated to overcome the “kinetic barrier” efficiently.

Most antitumor drugs were shown to induce immunosuppression and symptomatic toxicities (Yoshimatsu et al., 2008). In our study, the mice treated with DOX + VER exhibited severe side effects, including obvious weight loss (Figure 7B), WBC decrease (Table 3), hepatotoxicity and cardiotoxicity (Table 4). However, similar to the saline group, no obvious toxicities were observed in the CL-R8-LP (DOX + VER)/(+Cys) treated mice. Therefore, using liposomal drug delivery system may help reduce the side effects of DOX and VER. The improved bio-safety of CL-R8-LP (DOX + VER)/(+Cys) could be due to the increased accumulation in tumor and decreased distributions in normal tissues.

In this study, CL-R8-LP (DOX + VER) was successfully developed and exhibited an excellent anti-MDR tumor efficacy, while the drug combination used in this study just offered an example for application of the reduction-sensitive drug delivery. VER belongs to the first generation P-gp inhibitors, whose mechanism of action has already been studied clearly, thus using VER as a model drug is conducive to demonstrating the superiority of carrier. After that, the second or third generation inhibitors could also been encapsulated. For example, Patel et al. (2011) developed a long-circulating liposome to co-deliver tariquidar (XR9576) and paclitaxel, which could reverse the MDR efficiently. And Wang et al. (2012) simultaneously delivered GG918 (Elacridar) and DOX via Polymer-Lipid hybrid nanoparticles to enhance treatment of MDR breast cancer. In addition, we could co-encapsulate drugs that aim at different mechanisms of MDR to further enhance the reversal activity, such as the paclitaxel/lomustine-loaded epidermal growth factor receptor (EGFR)-targeted nanoparticles, which were developed to treat the MDR cancer via the effect of lomustine on the energy metabolism and “P-gp bypassing effect” of nanoparticles (Milane et al., 2011). Moreover, CL-R8-LP can be applied to the delivery of therapeutic gene and anti-MDR associated genes, which favors lysosomal escape.

Conclusions

In summary, the reduction-sensitive liposome co-encapsulating DOX and VER CL-R8-LP (DOX + VER) was successfully developed in this study. Under the synergy of “P-gp bypassing effect” and “P-gp inhibition effect”, the drug delivery system could overcome the P-gp over-expressed on the plasma membrane and nuclear envelope of MCF-7/ADR cells, and significantly improved the uptake efficiency, cytotoxicity and apoptosis and necrosis-inducing effect of DOX in MCF-7/ADR cells. In vivo studies showed CL-R8-LP (DOX + VER) could efficiently inhibit the growth of MCF-7/ADR tumor and reduce the toxicity of DOX and VER. Therefore, the CL-R8-LP (DOX + VER) provided us with a promising drug delivery strategy with an excellent anti-MDR tumor efficacy and high safety.

Declaration of interest

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