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RESEARCH PAPER

Bioactive Lipids-Based pH Sensitive Micelles for Co-Delivery of Doxorubicin and Ceramide to Overcome Multidrug Resistance in Leukemia

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ABSTRACT

Purpose Construction of a novel PEGylated bioactive lipids-based micelle system for co-delivery of doxorubicin (DOX) and short chain ceramide (C6-ceramide) to overcome multidrug resistance in leukemia.

Methods The PEGylated bioactive lipids-based micelle system was constructed via electrostatic and hydrophobic interactions among DOX, bioactive lipids PazPC and C6-ceramide. The micellar formulation was characterized in terms of size, zeta potential, stability and release behavior, etc., and *in vitro* cytotoxicity, *in vivo* antitumor efficacy and the underlying mechanism were further evaluated.

Results This novel micellar system showed small size (~15 nm), high drug encapsulation efficiency (>90%), good stability and endosomal acid-triggered release of DOX. Synergistic cytotoxic effects between DOX and bioactive lipid C6-ceramide in P-gp overexpressing drug resistant leukemia P388/ADR cells were observed. The mechanistic studies demonstrated that modulation of drug efflux system and induction of apoptotic effects by lipids were responsible for the synergistic effects between DOX and C6-ceramide in drug resistant leukemia P388/ADR cells. Using an *in-vivo* P388/ADR leukemia mouse model, the median survival time of the DOX-loaded PEGylated micelles with PazPC and C6-ceramide as major components was significantly greater than that of free DOX and control group.

Conclusions We developed a novel pH sensitive bioactive lipids-based micellar formulation which could potentially be useful in delivering chemotherapeutic drug DOX and provide a novel strategy to increase the therapeutic index for drug resistant leukemia treatment.

KEY WORDS ceramide · doxorubicin · leukemia · micelle · oxidized phospholipid

ABBREVIATIONS

DDS	Drug delivery system
DL%	Drug loading content
DLS	Dynamic light scattering
DOX	Doxorubicin hydrochloride
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylphosphatidyl choline
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
EE%	Encapsulation efficiency
ELS	Electrophoretic light scattering
ePC	Egg phosphatidylcholine
GCS	Glucosylceramide synthase
NBD-C6-ceramide	N-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine
OxPLs	Oxidized phospholipids
PazPC	1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine
PP/DOX	DOX-loaded PEG2000-DSPE/PazPC
PPC/DOX	DOX-loaded PEG2000-DSPE/PazPC/C6-ceramide
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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INTRODUCTION

Bioactive lipids, broadly defined, are category of lipids showing functional consequences when changes in lipid levels (1). Studies over the past decades have defined a panel of bioactive lipids, such as eicosanoids, glycerolipid-derived lipid, sphingolipids, oxidized phospholipids, and ceramides etc. As bioactive molecules, they play important roles in different signal transduction pathways, including anti- and pro-inflammatory effects, apoptotic effects, growth arrest, proliferation, survival and potent ligands for different G protein-coupled receptors (2,3). However, unlike some biologically inactive lipids, such as egg phosphatidylcholine (ePC), dipalmitoylphosphatidylcholine (DPPC) or distearoylphosphatidyl choline (DSPC) used as a well-characterized lipid based drug delivery systems for hydrophobic and/or hydrophilic drugs, bioactive lipids for drug delivery are still a relatively new concept in drug delivery field.

Oxidized phospholipids (OxPLs), as metabolic derivatives of phosphatidylcholine, are endogenous components on cell membrane and circulating lipid particles, constituting an important subclass of phospholipids and exhibiting unique physical and biological properties not found in their parent phospholipids (4,5). Recently, using an oxidized phospholipid PazPC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine), we fabricated a pH-sensitive micellar system via electrostatic and hydrophobic interaction between PazPC and anthracyclines for delivery of anticancer drugs (Fig. 1a–c) (6). This novel pH-sensitive micellar delivery system exhibited significantly enhanced DOX uptake and higher cytotoxicity effect on both sensitive and resistant leukemia cells (6). As an novel micellar system, OxPLs-based micelles exhibited highly desirable characteristics, such as small particle size (~10 nm), good stability at physiological conditions and pH-sensitive release, etc. (6). With these advantages, OxPLs-based micellar system could be a promising carrier that may improve the therapeutic outcomes of resistant leukemia. However, as a bioactive DDS assembled by a single lipid, PazPC/DOX micelles also have several limitations that may impede better antitumor efficacy in future animal studies or clinical trials. First, with respect to apoptotic effect induced by the bioactive DDS in tumor cells, PazPC itself as bioactive molecule just induced reversible mitochondria dysfunction and slightly apoptotic response in leukemia cells (7,8). Therefore, it is necessary to improve apoptotic effect induced by bioactive drug delivery carrier in tumors, especially in resistant tumors for better therapeutic outcomes. Second, the PazPC/DOX system assembled by a single bioactive lipid showed a relative low drug encapsulation efficiency (~60%) for DOX (6), which requires further development of the PazPC-based DDS to a mixed DDS using mixed bioactive lipids for potential antitumor application.

In order to address the potential limitations of the PazPC/DOX micellar system as above mentioned, the purpose of this work is to fabricate a new mixed micellar system, using an apoptosis-inducing lipid C6-ceramide, which may decrease the apoptosis threshold in tumor cells and improve drug encapsulation in the new micellar system. It has been reported that intracellular bioactive ceramides are generated by a series of chemotherapy drugs and act as tumor-suppressive lipids (9). However, in resistant cancer cells, ceramides generated by chemotherapy drugs are metabolized to glucosylceramides through up-regulating the gene expression of glucosylceramide synthase (GCS). This enzymatic process hastens clearance of ceramides and limits chemotherapy drug-induced apoptosis (3,10–13). Inspired by these facts, we hypothesize that co-delivery of ceramide with DOX to resistant tumor cell via a mixed OxPL-based micellar system may elevate the intracellular ceramide level and inhibit the activity of GCS through substrate inhibition. This strategy may decrease the apoptosis threshold and re-establish apoptotic effects induced by DOX in the resistant tumor cells. Furthermore, addition of hydrophobic lipid, C6-ceramide, in PazPC/DOX micelles could also improve the drug encapsulation efficiency.

To test this hypothesis, a bioactive lipid, C6-cermaide (Fig. 1d) was integrated into the PEGylated PazPC-based micellar system to form a new mixed micellar system (Fig. 1e), and *in vitro* and *in vivo* efficacy for overcoming drug resistance in leukemia was evaluated. Meanwhile, the underlying mechanisms of bioactive lipids in overcoming chemoresistance were also explored in this study.

MATERIALS AND METHODS

Materials

1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazPC) and DSPE-PEG2000 was obtained from Avanti Polar Lipids (Alabaster, AL). C6-ceramide and NBD-C6-ceramide were purchased from Cayman Chemicals (Ann Arbor, MI). Doxorubicin hydrochloride (DOX), HEPES, rhodamine123, verapamil, Sephadex G75 and PBS were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Fetal bovine serum and RPMI1640 medium were purchased from Mediatech (Manassas, VA), Murine leukemia cell lines, P388 and P388/ADR, were obtained from National Cancer Institute at Frederick (Frederick, MD).

Methods

Preparation and Optimization of PPC/DOX Micelles and PP/DOX Micelles

PPC/DOX micelle is an abbreviation of DOX-loaded PEG2000-DSPE/PazPC/C6-ceramide micelle, which was prepared using thin-film hydration method reported previously (6). Briefly, 50 μL PazPC (10 mg/ml), different volume of C6-ceramide (0–70 μl, 10 mg/ml) and DSPE-PEG2000 (0–100 μl, 10 mg/ml) in chloroform were pipetted into a glass tube, and the solvent was removed under a stream of nitrogen, and the lipid films were subsequently maintained under vacuum condition for at least 2 h. The dry lipid films were hydrated at 60°C for 30 min in 0.5 ml DOX solution (0.3 mM) in 20 mM HEPES buffer (pH 7.0) to yield different ratio of PEG2000-DSPE/PazPC/C6-ceramide. The DOX-loaded PPC micelles were finally sonicated in a bath-type sonicator for 10 min. PP/DOX micelle, as a control in subsequent experiments, is an abbreviation of DOX-loaded PEG2000-DSPE/PazPC micelle, which was prepared using the same procedure as above mentioned except for no C6-ceramide addition.

Characterization of PPC/DOX Micelles and PP/DOX Micelles

Particle size and zeta potential of the micelles were determined based on dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively, using a Nicomp Model 380/ZLS particle sizer (PSS, Santa Barbara, CA). The amount of DOX encapsulated in the micelles was determined using column separation method. Sephadex G75 packed in a disposable column (15 mm*70 mm, Thermo Scientific, Rockford, IL) was employed to separate the micellar DOX out of free DOX. 0.5 ml micelle sample was loaded into the column, and was eluted using 20 mM HPEPS buffer (pH 7.0) as mobile phase, and then fractions were collected. The concentrations of DOX in each collection tube were measured fluorometrically at 480 nm (excitation) and 590 nm (emission) using microplate reader (Fluostar, BMG labtechnologies, Germany). Elution profiles of DOX were then plotted *versus* elution volumes. The first peak reflects micellar drug, and the second peak reflects free drug. The encapsulation efficiency (EE%) and drug loading content (DL%) were calculated according to the previous study (6).

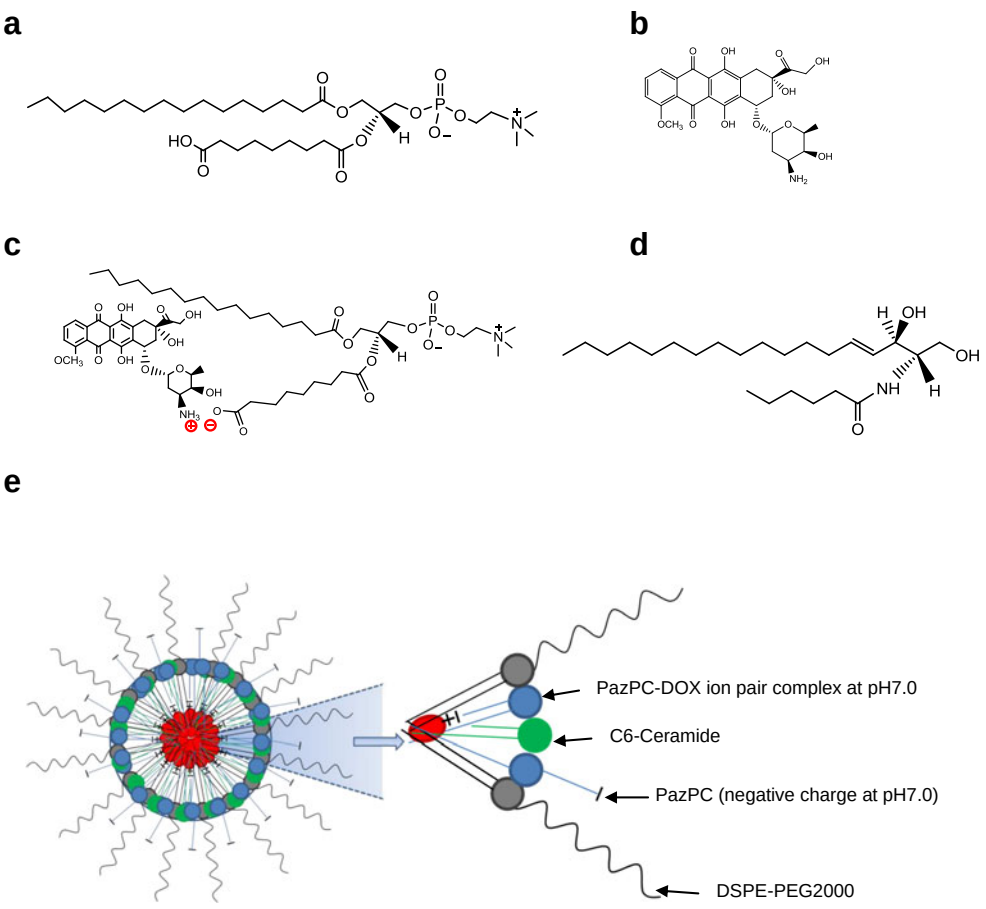

Fig. 1 Structures of bioactive lipid PazPC (**a**), antitumor drug DOX (**b**), PazPC-DOX ion-pair complex (**c**), bioactive lipid C6-ceramide (**d**) and PEGylated bioactive lipids-based PPC/DOX micelle (i.e. DOX-loaded PEG2000-DSPE/PazPC/C6-ceramide micelle) (**e**).

Stability of PPC/DOX Micelles Under Different Conditions

The stability of PPC/DOX micelles was evaluated at room temperature, acidic pH and in physiologically ionic strength. The PP/DOX micelle without C6-ceramide was used as a control. For the storage stability, the micellar DOX at pH 7.0 were freshly prepared, and then stored at room temperature (~25°C) for 6 days, finally applied to Sephadex G75 column, and eluted with 20 mM HEPES buffer at pH7.0. For the stability in acidic pH condition, the pH of the freshly prepared sample was adjusted to 6.0 or 5.0 using 0.6 N HCl, incubated at 37°C for 30 min, then applied to Sephadex G75 column, and eluted with 20 mM HEPES buffer at corresponding pH. For the micelle stability in physiologically ionic strength, proper aliquots of 25×PBS were first added into the micelle solution to yield a final concentration of 1×PBS. Then, the salted sample was incubated at 37°C for 30 min, applied to Sephadex G75 column, and finally eluted with 1× PBS-containing 20 mM HEPES buffer. Elution profiles of DOX from all samples were plotted *versus* elution volumes according to the fluorescence ($\lambda ex = 480$ and $\lambda em = 590$) of DOX fraction in collection tubes. The encapsulation efficiency, which reflects the stability of the micelles under different conditions, was calculated according to the previous study (6).

Release Behavior of DOX from PPC/DOX Micelles at Different pHs

DOX release from the PPC/DOX micelles was studied in different pH buffers. The PP/DOX micelle was used as a control. Briefly, sink condition was established by placing 1 ml of DOX-loaded micelles or free drug solution ([DOX] = 0.3 mM in each sample) in dialysis tubes (MWCO 15 K, Spectra/por membrane tubing, Spectrum Labs, CA), and then by immersing the dialysis tubes in large volume centrifuge tubes containing 40 ml of release buffers with different pH (1×PBS, pH 7.4; 0.1 M citrate buffer, pH 6.0 or 5.0). All the centrifuge tubes were incubated at 37°C under mild agitation. 0.5 ml of dialyzate sample was collected at different time intervals and the same volume of fresh release medium was replenished immediately. The concentration of DOX in dialyzate was analyzed fluorometrically at 480 nm (excitation) and 590 nm (emission), and cumulative release profiles were then plotted verse release times.

Cytotoxicity of PPC/DOX Micelles Against Leukemia P388 and P388/ADR Cells

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay was utilized to assess cytotoxicity of the PPC/DOX micelles. The PP/DOX micelle, blank PPC and PP micelles, free C6-ceramide in 1‰ DMSO and free DOX were used as controls. Both cells were seeded in 96-well plates at 5,000 cells in 100 μl RPMI1640 medium with 10% FBS/well. Serial dilutions of micellar DOX were added to the plate at 37°C in 5% CO_2 for 48 h. 10 μl of MTT stock solution (5 mg/mL in PBS; pH 7.4) was then added into the wells and the plates were incubated at 37°C for another 4 h. Cell suspensions were spun down for 10 min at 1,000 rpm. The medium was removed and 100 μl DMSO was then added to each well to solubilize the dye. The absorbance was measured using microplate reader (Fluostar, BMG labtechnologies, Germany) at 540 nm, and the concentration of drug that inhibited cell survival by 50% (IC_{50}) was determined from cell survival plots using "DoseResp" function of OriginPro 8.0.

DOX Uptake in Leukemia P388 and P388/ADR Cells

The cellular uptake of micellar DOX or free DOX was first visualized by a fluorescent microscope (EVOS fl Microscope, AMG, USA). Briefly, P388 and P388/ADR cells were seed into 12-well plates at a cell density of 10^6 cells/ml and a proper volume of micellar DOX or free DOX was then added into 12-well plate to obtain 10 μM DOX concentration. After 2 h incubation, the cells were fixed with fixation buffer (4% paraformaldehyde, Biolegend) for 20 min at room temperature. Cellular uptake of micellar DOX or free DOX was finally visualized under the light channel of RFP.

The extent of cellular uptake of micellar DOX or free DOX was further quantitatively determined using flow cytometry (BD Accuri C6 Cytometer, San Diego, CA) by measurement of the cell associated fluorescence. Briefly, P388 and P388/ADR cells were seeded on 12-well plates at cell density of 10^6cells/ml. 10 μM of micellar DOX or free DOX was given to the cells and incubated at 37°C for 2 h. The fluorescence was then measured with flow cytometry by collecting 20000 events for each sample. Each experiment was performed in triplicate.

Rhodamine 123 Uptake and Efflux Studies

For Rho 123 uptake study, 1×10^6 P388 or P388/ADR cells in 12-well plate was first incubated in serum-free RPMI 1640 medium at 37°C, 5% CO_2 for 10 min, then 1 μM free Rho123, or 1 μM Rho123/blank micelles at different concentrations (50, 10, 1 and 0.1 μM), and 1 μM Rho123/50-μM verapamil (Vrp) were added to the medium, and the plate was incubated for another 30 min. The cells were placed on ice, spun down at 1,000 rpm, 4°C for 3 min, and washed twice using PBS. The cell associated Rho123 fluorescence was analyzed with flow cytometry.

For Rho 123 efflux study, 1×10^6 cells were first loaded with 1 μM free Rho123, or 1 μM Rho123/50 μM blank micelles, and 1 μM Rho123/50 μM Vrp for 30 min at 37°C. The cells were placed on ice, spun down at 1,000 rpm, 4°C for

3 min, washed twice using PBS, and resuspended in Rho123-free RPMI 1640 media. For Rho123/blank micelles and Rho123/Vrp group, the Rho123-free media contained respective blank micelles or Vrp at the same concentrations that were used for the loading. The loaded cells in the Rhol23-free medium were incubated at 37°C, 5% CO_2 for 0.5-2 h, and at predetermined time point, the cell associated Rho123 fluorescence was analyzed with flow cytometry.

Apoptosis Detection

The TUNEL assay was used to detect apoptosis in P388 or P388/ADR cells. Briefly, both cells was incubated with 50, 10, 1 and 0.1 μM of blank micelles in 10% serum RPMI 1640 at 37°C, 5% CO_2 for 16 h, then the cells were added to the poly-L-lysine-coated slides, dried in hood for 15 min. Slides were immersed in 4% formaldehyde for 25 min, and cells were permeabilized with Triton X-100 for 5 min and then processed for the dUTP nick end labeling according to manufacturer's protocol (DeadEnd™ Colorimetric TUNEL system, Promega). The stained slices were finally observed with a phase contrast microscope, and the apoptotic nuclei were stained dark brown. The images were captured by a digital camera.

Intracellular Distribution

The 10% NBD-C6-ceramide labeled PPC/DOX micelles was first prepared in order to visualize the intracellular distribution of the blank PPC micelles and the drug. 1×10^6 P388/ADR cells in 12-well plate was incubated in 10% serum RPMI 1640 medium with the NBD-labeled micellar DOX (10 μM) at 37°C, 5% CO_2 for 15 min–2 h, and the cells were also incubated with 4 μM Hoechst 33342 for another 15 min prior to visualization. At the predetermined time point, the cells were spun down at 1,000 rpm for 3 min, washed 2 times using PBS, then fixed with 4% (w/v) formaldehyde in PBS (pH 7.4) for 20 min at room temperature. The fluorescence was visualized with EVOS fl microscope under the light channel of RFP (for DOX), GFP (for NBD-labeled micelles) and DAPI (for Hoechst 33342).

In Vivo Study

Five-week-old male CD2F1 mice were used to test *in vivo* efficacy of micellar DOX. The animal experiments complied with the rules set forth in the NIH Guide for the Care and Use of Laboratory Animals and IACUC committee of authors' institutes. 1×10^5 P388/ADR cells were implanted intraperitonaeally (i.p.) in each mouse. The day of tumor cell inoculation was designated as Day 0. The animals were randomly assigned to various treatment groups: PPC/DOX micelles, PP/DOX micelles, blank PPC micelles, free DOX and PBS control. Each group had 8 mice. On Day 1 after tumor implantation, the micellar DOX or the controls were injected i.p. at the dose of 3.0 mg DOX/kg/injection on the q4d×3 treatment schedules. The median survival time (mST) was recorded, and the increase in life-span (ILS) based on the mST was calculated as follows (14):

$$\%ILS = \frac{mST\ of\ a\ treatment\ group - mST\ of\ the\ control\ group}{mST\ of\ the\ control\ group} \times 100\%$$

Statistical Analysis

The data were expressed as mean ± S.D. Statistical significance in cytotoxicity, uptake and efflux studies were determined using one-way ANOVA followed by a Student's *t* test for multiple comparison tests. Differences in survival rate in treatment groups of *in vivo* study were analyzed by Kaplan-Meier survival analysis. P value of <0.05 is considered as statistically significant.

RESULTS

Preparation and Characterization of PPC/DOX Micelles

PPC/DOX micelle (an abbreviation of DOX-loaded PEG2000-DSPE/PazPC/C6-Ceramide micelle) is defined as a PEGylated stealth bioactive micelle that was prepared by adding DSPE-PEG2000 and C6-Ceramide to PazPC/DOX micelles using the thin-film procedure. Based on the PazPC/DOX micelle formulation previously optimized in our lab (6) and a series of preliminary studies (data not shown), 20% DSPE-PEG2000 and 31% C6-ceramide (mol/mol) were chosen to make the lipid mixture and form a film. After hydration, a stable mixed micellar formulation was successfully obtained. The molar ratio of the final optimized PPC/DOX micelles is 2.2/5/3.3/1 for PEG2000-DSPE/PazPC/C6-Cermaide/DOX, respectively ([DOX] = 0.3 mM in 20 mM HEPES buffer, pH 7.0). The PP/DOX micelles without C6-ceramide (an abbreviation of DSPE-PEG2000/PazPC/DOX micelles), the blank PPC micelle (an abbreviation of blank PEG2000-DSPE/PazPC/C6-Ceramide micelle) and blank PP micelle (an abbreviation of blank PEG2000-DSPE/PazPC micelle) were also prepared at the respective molar ratios for control purposes in subsequent experiments. The characterization of resultant micelles is summarized in Table I and Fig. 2. The particle sizes of the PPC/DOX micelles, the PP/DOX micelles, blank PPC micelles and blank PP micelles were in the range of 12–16 nm. DOX encapsulation process or addition of C6-Cermaide in the micelles didn't significantly increase the particle size.

Table I also shows the DOX micelles had a lower zeta potential than the blank micelles, which was consistent with the data reported previously (6). A lower zeta potential in the micellar DOX suggested that the ion-pair complexes were formed between the carboxyl group of PazPC and the primary amino group of the glycosidyl residue in DOX. Similar DOX encapsulation efficiency was also observed in the PPC/DOX micelles compared to the PP/DOX micelles, indicating that C6-ceramide in the micelles did not interfere with the DOX entrapment in the core by hydrophobic interaction.

Stability of the Miceller DOX at Room Temperature, Lower pH, and in PBS

The stability of PPC/DOX micelles and PP/DOX micelles was studied using G75 size exclusion chromatography. This method can easily separate the free drugs from micelles (6). Figure 3 shows the stability of DOX-loaded PPC micelles and PP micelles under different conditions: room temperature storage, acidic pH, and physiological salt concentration. The micelle stability was reflected by the change of its elution profiles after it was passed through Sephadex G75 column. The first peak of the elution profiles reflects the DOX associated with micelles and the second peak reflects the free DOX in the micellar formulation.

As shown in Fig. 3a–b, compared to day 1, there is no obvious changes in elution profiles of the micelles after the micelles were stored at room temperature for 6 days. The data of encapsulation efficiency of the micelles in day 6 showed the same as day 1, ~93% for the PPC/DOX micelles and ~91% for the PP/DOX micelles (Table II). When the micelles were incubated at pH 6.0 or pH 5.0 for 30 min, DOX was significantly dissociated from the micelles, resulting in the area of the first peak dramatically decreased and the second peak increased (Fig. 3c–d). The encapsulation efficiency of the PPC/DOX micelles was ~60% at pH 6.0, ~40% at pH 5.0, compared to ~93% at pH7.0. As a control, the encapsulation efficiency of the PP/DOX micelles at acidic pH also shows similar decrease (~52% at pH6.0, ~26% at pH5.0) compared to ~91% at pH7.0 (Table II). Therefore, like the PazPC/DOX micelles (6), incorporation of C6-ceramide into the micelles didn't change the pH-responsive property and the PPC/DOX micelles was still pH-sensitive. When the micelles were incubated in 1×PBS buffer for 30 min, the second peak of the PPC/DOX micelles and the PP/DOX micelles increased just slightly (Fig. 3e–f), and encapsulation efficiency of the micelles were maintained at ~90% for the PPC/DOX micelles or 88% for the PP/DOX micelles (Table II). That indicates only small amount of drug dissociated from the micelles, and they were relatively stable in physiological salt concentration.

In Vitro pH-Sensitive Release of DOX from the Mixed Micelles

The pH-dependent stability of the micelles was demonstrated in Fig. 3c–d, so the release profiles of this system under different pH values were further investigated in detail. As shown in Fig. 4, a control experiment with free DOX confirmed that the high MWCO (15 K) of dialysis membrane tubing couldn't restrict diffusion of the released drugs, and they were able to reach 100% release after 3.5 h. However, the release of DOX from the PPC/DOX micelles and the PP/DOX micelles at different pH couldn't reach a plateau until at least 10 h. The total released drug from the PPC/DOX micelles was significantly different under different pH conditions. Up to 43.6% of total drug were released at the physiological pH 7.4 and around 85.1% and 91.4% of total drug were released at pH 6.0 and 5.0, respectively. Importantly, the release rate of drug from both micelles increased with decreasing of the pH of release medium. Interestingly, at any given pH, the total release of DOX from the PP/DOX micelles displayed a slightly higher rate than the PPC/DOX micelles during 24 h. This indicated that C6-ceramide in the PPC/DOX micelles increased the DOX affinity to the core probably by hydrophobic interaction, which led to a lower release rate of DOX in release media.

Table I Characterization of PPC/DOX Micelle and PP/DOX Micelle

	Particle Size (nm)	Zeta Potential (mV)	Encapsulation Efficiency (%)	Drug Loading (%)
Blank PP micelle	12.9 ± 2.5	−20.2 ± 6.3	–	–
PP/DOX micelle	13.1 ± 2.5	−8.4 ± 1.1	91.28	12.6
Blank PPC micelle	14.2 ± 2.8	−21.0 ± 5.1	–	–
PPC/DOX micelle	15.8 ± 2.9	−6.7 ± 0.89	93.36	8.2

PP/DOX micelle is an abbreviation of DOX-loaded PEG2000-DSPE/PazPC micelle, *PPC/DOX micelle* is an abbreviation of DOX-loaded PEG2000-DSPE/PazPC/C6-ceramide micelle, *blank PP micelle* is a counterpart of PP/DOX micelle without loading DOX, *blank PPC micelle* is a counterpart of PPC/DOX micelle without loading DOX, For the molar ratio of components in PPC/DOX or PP/DOX micelle, PazPC/C6-Ceramide/DOX = 5/3.3/1; [DOX] = 0.3-mM, and [DSPE-PEG2000] in total lipid = 20%, mol/mol

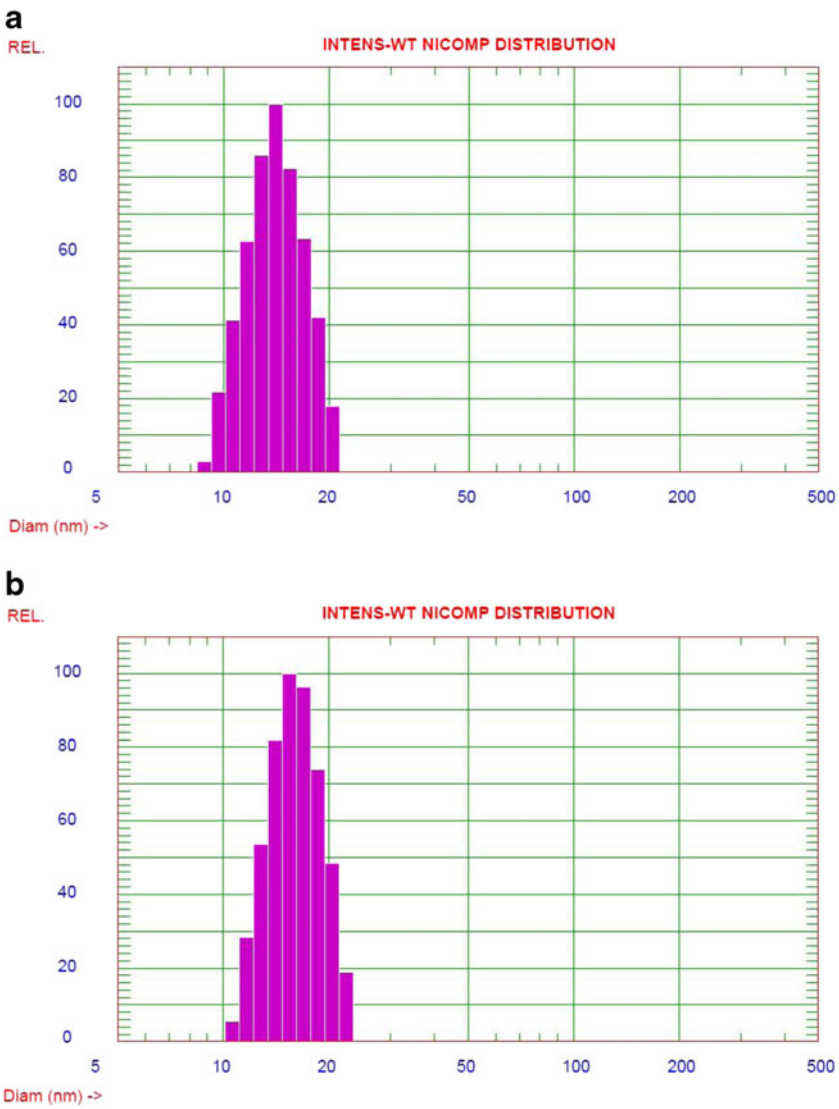

Fig. 2 Representative Nicomp distribution analysis (intensity-wt) of the blank PPC micelles (**a**, 14.2 ± 2.8 nm) and PPC/DOX micelles (**b**, 15.8 ± 2.9 nm) measured by DSL. The components and their molar ratios of the PPC/DOX micelle and blank PPC micelle refer to the Notes of Table I.

Enhanced Cytotoxicity of the Mixed Micelles in Leukemia Cells *in Vitro*

In vitro cytotoxicity of the PPC/DOX micelles, the PP/DOX micelles, free DOX, free C6-ceramide, blank PPC and blank PP micelles were conducted in the sensitive leukemia cells P388 and resistant P388/ADR cells. The IC_{50} values were listed in Table III, and cell viability of both leukemia cells were shown in Fig. 5. The PPC/DOX micelles and the PP/DOX micelles exhibited significantly higher cytotoxicity ($P<0.01$) in both cells compared to free DOX, free C6-ceramide and the blank PPC micelles, respectively. First, compared to free DOX, the IC_{50} values of the PPC/DOX micelles were 21-fold lower in resistant P388/ADR cells and 16-fold lower in P388 cells; the PP/DOX micelles were 9-fold lower in resistant P388/ADR cells and 8-fold lower in the sensitive P388 cells. Moreover, compared to free C6-ceramide or the blank PPC micelles, the IC_{50} values of the PPC/DOX micelles were 7–8 folds lower in P388/ADR cells and 51–60 folds lower in P388 cells. This indicates that bioactive lipid C6-ceramide and antitumor drug DOX in the PEGylated PazPC micelles exerted synergistic toxic effects, which led to the IC_{50} values 7–21 folds lower in P388/ADR cells and 16–60 folds lower in P388 cells. Interestingly, the cytotoxicity of PPC/DOX in resistant cells was increased to the point where the sensitive cells responded to free DOX, resulting in the comparable IC_{50} values of the same order of magnitude (226 nM *vs* 215 nM, Table III).

Intracellular Traffic and Distribution of DOX and C6-Ceramide in P388/ADR Cells

In order to understand the cellular mechanism of enhanced cytotoxicity in resistant cells generated by DOX-loaded bioactive lipids-based micelles, we first incorporated fluorescence (NBD) labeled C6-ceramide into the micelles and then monitored the intracellular distribution of C6-ceramide and DOX by a fluorescence microscopy for 2 h treatment with

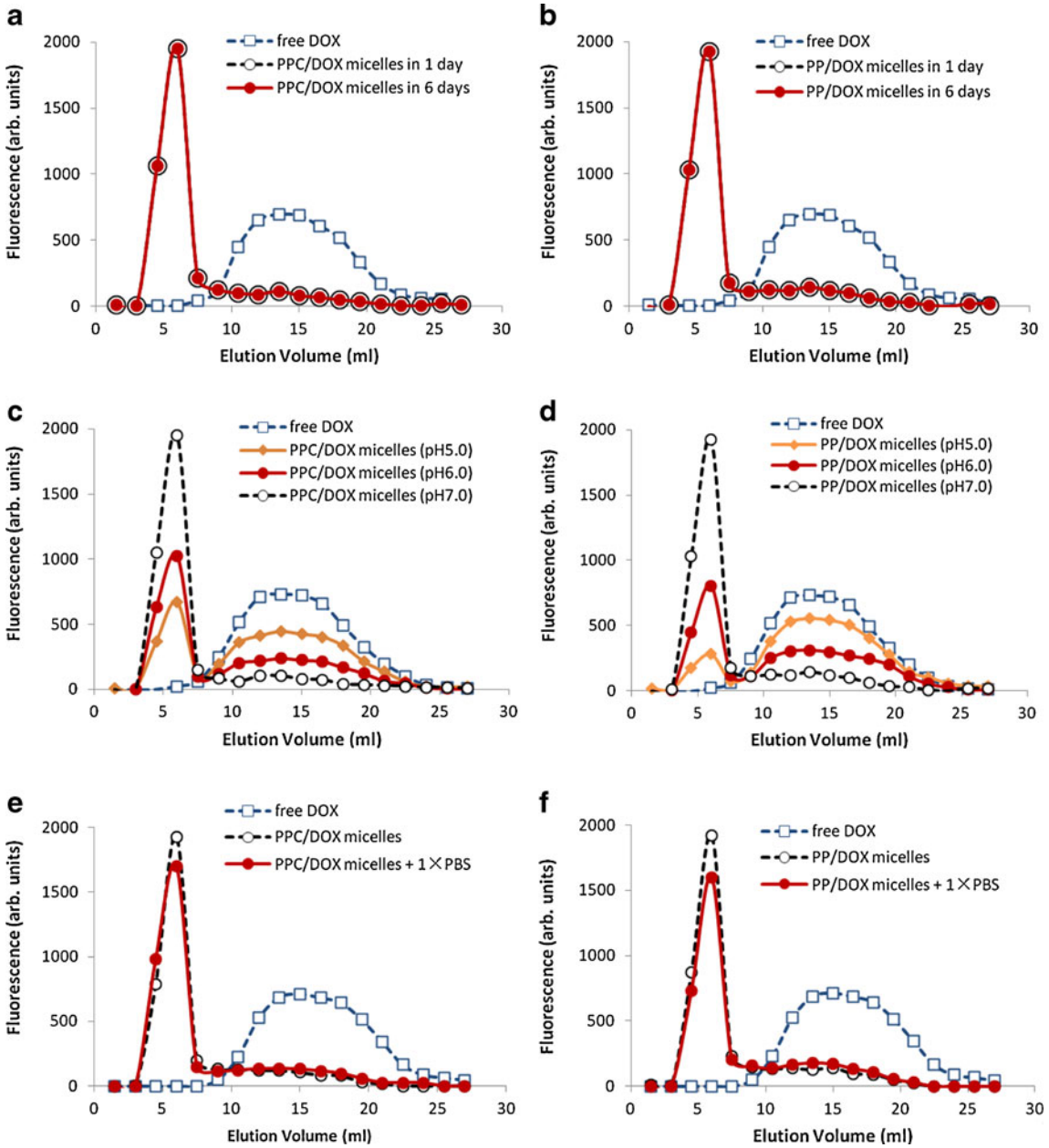

Fig. 3 *In vitro* stability of the PPC/DOX micelles and the PP/DOX micelle under different conditions: room temperature in 6 days (**a**–**b**), acidic pH (**c**–**d**), 1 × PBS (**e**–**f**). The components and their molar ratios of the PPC/DOX micelle and PP/DOX micelle refer to the Notes of Table I.

Table II Encapsulation Efficiency (EE%) of the Micellar DOX Under Different Conditions

	pH = 7.0			pH = 6.0[c]	pH = 5.0[c]
	1 day[b]	6 days[b]	1 × PBS[c]		
PP/DOX micelle[a]	91.28 ± 1.51	91.65 ± 2.14	88.32 ± 1.45	52.13 ± 2.16	26.87 ± 2.39
PPC/DOX micelle[a]	93.36 ± 0.98	93.21 ± 1.79	90.81 ± 0.68	60.69 ± 1.58	40.12 ± 3.21

[a] The components and their molar ratios of the PP/DOX micelle and PPC/DOX micelle refer to the Notes of Table I

[b] Normal micellar DOX formulation was placed at RT in 20 mM HEPES buffer (pH7.0), without 1 × PBS

[c] Fresh preparations of micellar DOX exposed in specific buffers for 30 min at 37°C

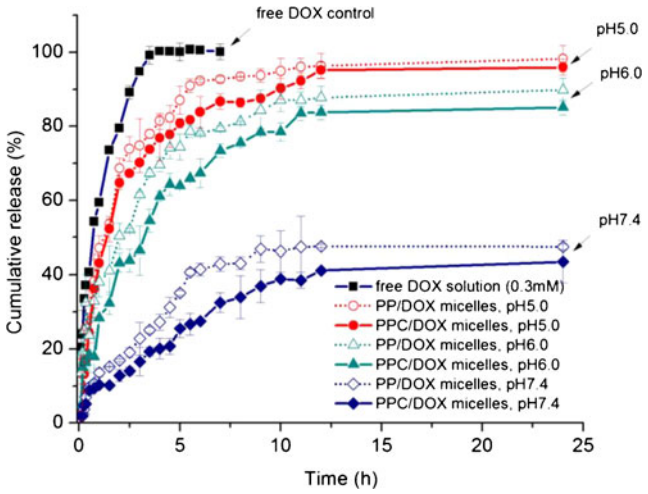

Fig. 4 *In vitro* release profiles of DOX from PPC/DOX micelles and PP/DOX micelles at different pH and at 37°C. The data were expressed by plotting cumulative release verse release times. The free DOX was completely released during 3.5 h, and during 24 h the cumulative DOX release of 43.6% and 47.5% from PPC/DOX micelles and PP/DOX micelles at pH7.4, 85.1% and 89.9% at pH6.0, and 91.4% and 93.6% at pH 5.0 were observed, respectively. The components and their molar ratios of the PPC/DOX micelle and PP/DOX micelle refer to the Notes of Table I.

the PPC/DOX micelles. As shown in Fig. 6, similar pattern of DOX and C6-ceramide distribution in the cytoplasm, but not in the nucleus of P388/ADR cells was observed at the early period, such as 15–30 min after the cells were exposed to the PPC/DOX micelles. The majority of orange fluorescence (DOX) and the green fluorescence (NBD-C6-ceramide) co-localized outside of the nucleus. During 30 min −1 h, the distribution of DOX and C6-ceramide gradually became distinct. At 1 h time point, DOX was partially separated from C6-ceramide and distributed in the nucleus, but the C6-ceramide was still in the cytoplasm. At 2 h time

Table III IC_{50} Values of Free DOX, PP/DOX Micelles and PPC/DOX Micelles on Leukemia Cells (48 h)

	P388	P388/ADR
Free DOX	215.43 ± 46.25	4887.97 ± 456.32
PP/DOX micelles[a]	24.65 ± 6.43*	523.38 ± 66.32*
PPC/DOX micelles[a]	13.36 ± 1.65*	226.78 ± 38.24*
Blank PPC micelles[a,b]	687.71 ± 51.23**	1708.71 ± 103.26**
Free C6-cermaide in 1‰ DMSO[b]	811.76 ± 72.64**	1669.79 ± 126.25**

IC_{50} was determined from cell survival plots using "DoseResp" function of OriginPro 8.0;

[a] The components and their molar ratios of the PP/DOX micelle, PPC/DOX micelle, and blank PPC micelle refer to the Notes of Table I

[b] The concentration of blank micelle or free C6-cermaide refers to "drug equivalent" concentration

*$P < 0.01$, as compared with free drug

**$P < 0.01$, as compared with PPC/DOX micelle

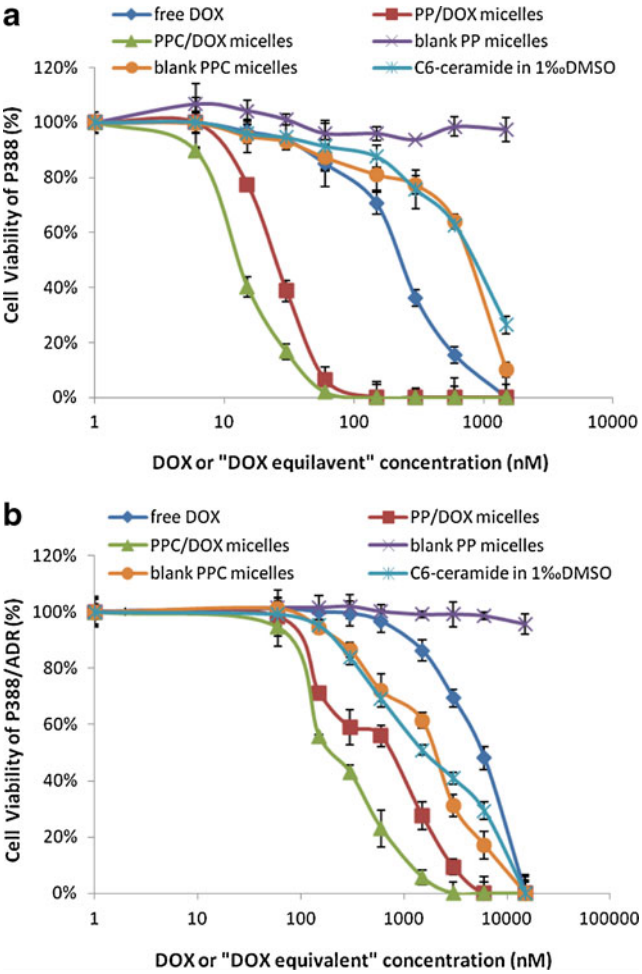

Fig. 5 Cell viability of P388 (**a**) and p388/ADR (**b**) leukemia cells treated with the PPC/DOX micelle or the PP/DOX micelles for 48 h. Cells were seeded in 96-well plates at 5,000 cells/well in 100 μl RPMI 1640 medium with 10% FBS, then incubated with the micellar DOX at different concentration at 37°C, 5% CO_2 for 48 h, and the viable cells were finally quantified using MTT assay. The free DOX, the blank micelles or free C6-Ceramide in1‰ DMSO were used as controls. The components and their molar ratios of the PPC/DOX micelle, PP/DOX micelle, blank PPC micelle and blank PP micelle refer to the Notes of Table I.

point, exact overlay between the orange and blue fluorescence was observed indicating the accumulation of the majority of DOX in the nucleus, but the C6-ceramide (green fluorescence) was still located in the cytoplasm of P388/ADR cells.

Enhanced DOX Uptake by the Mixed Micelles in Leukemia P388 and P388/ADR Cells

The uptake of the PPC/DOX micelles containing 10 μM of DOX was examined in sensitive P388 and resistant P388/ADR cells. The PP/DOX micelles and free DOX were used as controls. The uptake of DOX by leukemia cells was investigated using a flow cytometry by measurement of cell-associated fluorescence intensity. After a period of 2 h incubation, the uptake of DOX was significantly increased

by the PPC/DOX micelle formulation compared to free DOX and the PP/DOX micelles in P388 cells and resistant P388/ADR cells (Fig. 7a–c). The cellular uptake of DOX was also examined in sensitive P388 and resistant P388/ADR cells with fluorescence microscope. After a period of 2 h incubation, the enhanced uptake of the PPC/DOX micelles was observed in both leukemia cells compared to free DOX and the PP/DOX micelles. Figure 7d–f presents the typical fluorescence images of enhanced DOX uptake in resistance P388/ADR cells by the PPC/DOX micelles at the DOX concentration of 10 μM, compared to free DOX and the PP/DOX micelles.

Bioactive Lipids Inhibited P-gp Function in P388/ADR Cells

The effect of blank PPC micelles or blank PP micelles on Rho123 influx and efflux in P388/ADR cells was further evaluated. As shown in Fig. 8a, the blank PPC micelles and PP micelles significantly enhanced the accumulation of Rho123 in resistant P388/ADR cells in the concentration range of 0.1–50 μM. At the concentration of 50 μM, the blank PPC micelles dramatically enhanced 8.3-fold uptake of Rho 123, which was higher than 50 μM Vrp (6.0-fold increase). But at the same concentration, the blank PP micelles only increased 2.2-fold uptake of Rho123. At the concentration range of 0.1–10 μM, the blank PPC micelles also showed a slightly higher enhancement of Rho123 accumulation in P388/ADR cells. Meanwhile, a fast component in the efflux kinetic rate was observed in the case of free Rho123 from the results of kinetics of Rho123 efflux in P388/ADR cells (Fig. 8b). Compared to free Rho123, the rate of Rho123 efflux was much slower when both blank micelles were used. Interestingly, the blank PPC micelles also showed a slightly lower efflux rate of Rho123 than the PP micelles. These results indicate that C6-ceramide in the micelles inhibits P-gp activity more potently than PazPC in P388/ADR cells although both blank micelles blocked P-gp mediated rapid drug efflux, which led to higher Rho123 accumulation in resistant P388/ADR cells.

Bioactive Lipids Induced Apoptosis in P388/ADR Cells

To determine if the blank PPC micelles and the blank PP micelles induce apoptosis and nuclear DNA fragmentation in leukemia cells, TUNEL colorimetric assay (Terminal

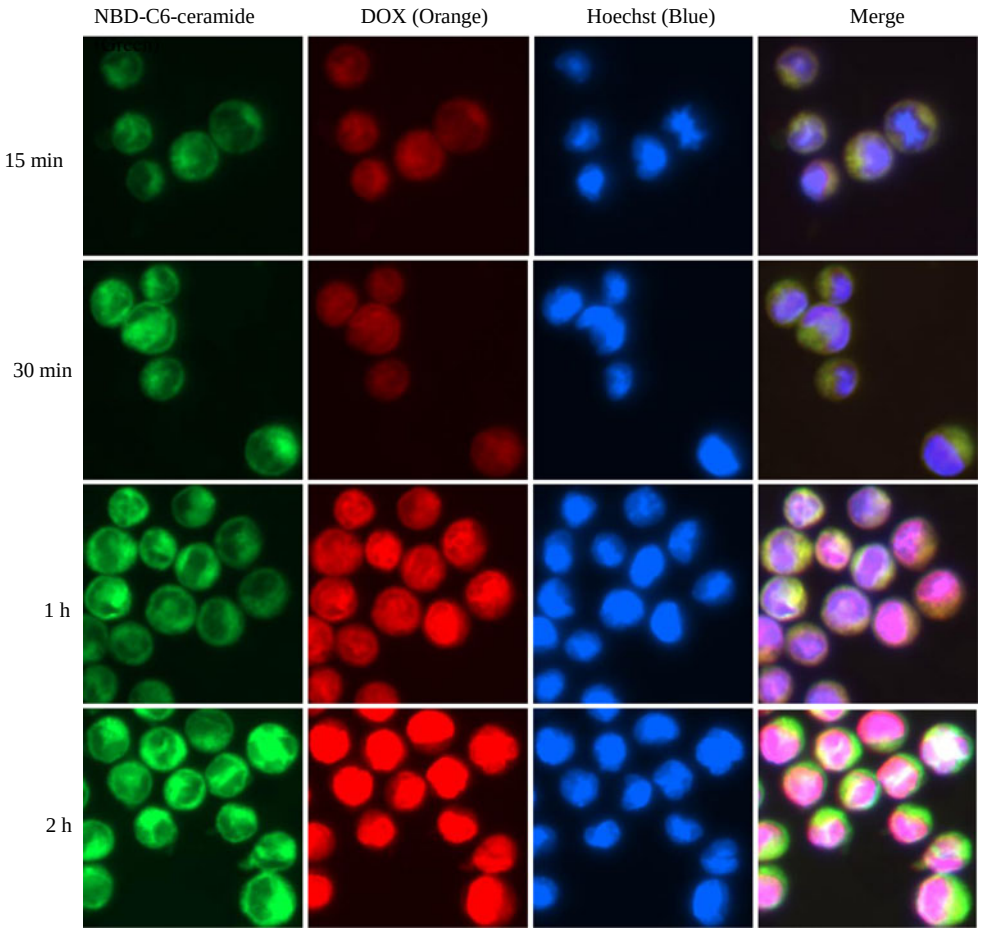

Fig. 6 Time course of intracellular distributions of DOX and C6-ceramide in P388/ADR leukemia cells treated with the PPC/DOX micelles at the DOX concentration of 10 μM. The PPC/DOX micelles were labeled with 10% NBD-C6-ceramide. P388/ADR cells were incubated in 10%-serum RPMI 1640 medium with the NBD-labeled micellar DOX at 37°C, 5% CO_2 for 15 min–2 h, and the cells were also incubated with 4 μM Hoechst 33342 for another 15 min prior to visualization. At the predetermined time point, the cells were fixed with 4% (w/v) formaldehyde, and then visualized with EVOS fl microscope under the light channel of RFP (for DOX), GFP (for NBD-labeled micelle) and DAPI (for Hoechst 33342). The components and their molar ratios of the PPC/DOX micelle refer to the Notes of Table I.

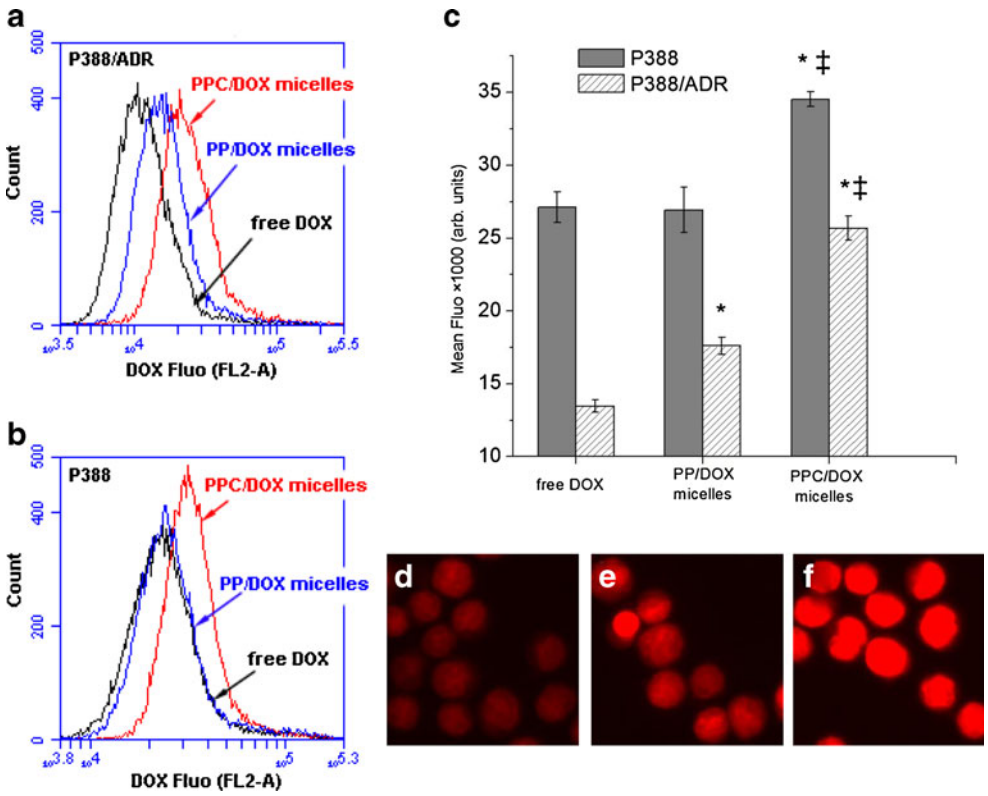

Fig. 7 Cellular uptake of the PPC/DOX micelles in P388 and/or P388/ADR cells. (**a**–**c**) Cellular uptake analysis by flow cytometry through treating cells with the PPC/DOX micelles at the DOX concentration of 10 μM for 2 h; free DOX and the PP/DOX micelles were used as controls. (**a**) Comparison of fluo histogram of DOX uptaken by P388/ADR cells; (**b**) Comparison of fluo histogram of DOX uptaken by P388 cells; (**c**) Statistical analysis of mean fluo of DOX uptaken by both cells, $^{*}p < 0.01$, as compared with free drugs in respective cells, $^{\ddagger}p < 0.01$, compared with the PP/DOX micelles in respective cells; (**d**–**f**) Cellular uptake in P388/ADR cells visualized by fluorescence microscope with treatment of 10 μM DOX for 2 h. (**d**) Free DOX, (**e**) PP/DOX micelles, (**f**) PPC/DOX micelles. The components and their molar ratios of the PPC/DOX micelle and PP/DOX micelle refer to the Notes of Table 1.

deoxynucleotidyl transferase Biotin-dUTP Nick End Labeling) was performed. Both cells were treated with the PPC micelles and the PP micelles for 16 h. In cells that are positive for apoptosis, nuclei are stained brown. As shown in Fig. 9, both blank micelles induced apoptosis of P388 and P388/ADR cells in the range of 1–50 μM concentration, but the PPC micelles resulted in more apoptotic cells than the PP micelles at the same concentration. Interestingly, in comparison between resistant and sensitive cells, no obvious difference in the rate of apoptosis was observed at a given

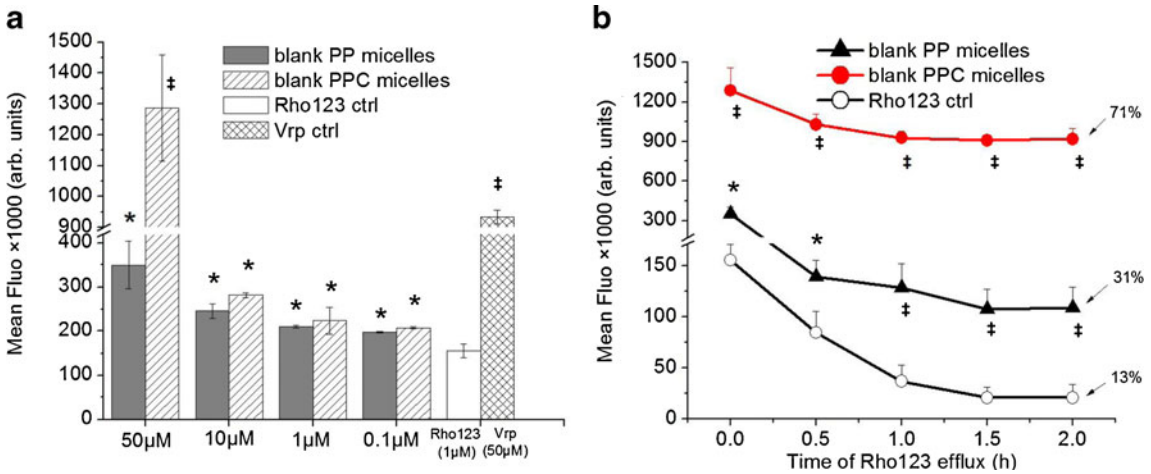

Fig. 8 The effect of blank PPC micelles on Rho123 influx and efflux in P388/ADR cells. (**a**) Intracellular accumulation of 1.0 μM Rho123 in P388/ADR cells cultured in serum-free medium in the presence of 50, 10, 1 and 0.1 μM blank PPC micelles at 37°C, 5% CO_2 for 30 min. (**b**) Efflux rate of Rho123 in P388/ADR cells. The cells was first treated with 1.0 μM Rho123/50 μM blank PPC micelles at 37°C, 5% CO_2 for 30 min; after being centrifuged, the cells were immediately resuspended in Rho123-free medium in the presence of 50 μM blank micelles and were incubated at 37°C, 5% CO_2 for 0.5–2 h. The blank PP micelles at the same concentration were used as a control. The cell associated Rho123 fluorescence was analyzed with flow cytometry at predetermined time point by collecting 20000 events for each sample. $^{*}P < 0.05$, and $^{\ddagger}P < 0.01$, compared with free Rho123. The components and their molar ratios of the blank PPC micelle and PP micelle refer to the Notes of Table 1.

concentration. In both cells treated with the blank PPC micelles at the 10 and 50 μM concentration, the rate of apoptotic cells was >90%, whereas ~20–40% of DNA fragmentation in cells was observed when cells were treated with the blank PP micelles at the same concentration. At the concentration of 1 μM, about 40% rate of apoptosis was observed in both cells when treated with the PPC micelles, but the rate is <5% for the PP micelles in both cells. Moreover, there was no difference in apoptosis between the cells treated with 0.1 μM of blank micelles and untreated cells, which indicated that 0.1 μM of blank micelles was not effective to induce cell apoptosis.

In Vivo Animal Efficacy Study

The *in vivo* efficacy study of DOX in the bioactive lipids-based micelles was performed in the murine P388/ADR leukemia model in male CD2F1 mice at DOX dose of 3 mg/kg/injection given with the treatment schedule of q4d×3. As shown in Fig. 10, compared to PBS control group (mST = 13 days), this *in vivo* treatment resulted in the median survival time (mST) of 25 days and the increase in life-span (ILS) of 92% for the PPC/DOX micelles, 16.6 days and 27% for the PP/DOX micelles, 16 days and 23% for free DOX, 12 days and −7% for the blank PPC micelles, respectively. Interestingly, in this *in vivo* animal study, there was no survival difference between PBS control and blank PPC micelles with the equivalent C6-ceramide concentration of the DOX-loaded PPC micelles. The PP/DOX micelles also showed slightly higher effective than free DOX (27% *versus* 23% of ILS). However, the PPC/DOX micelles, showing 25-day mST and 92% ILS, was found to be statistically significant when compared with the PP/DOX micelles, free DOX, the blank PPC micelles. This indicates that there was synergistic effect between DOX and C6-ceramide when co-delivered via the PEGylated bioactive lipids-based micellar system into the murine P388/ADR leukemia model in male CD2F1 mice.

DISCUSSION

The structure of C6-ceramide contains a sphingosine base, 6-carbon fatty acid chain at the N-2 position and hydroxyl

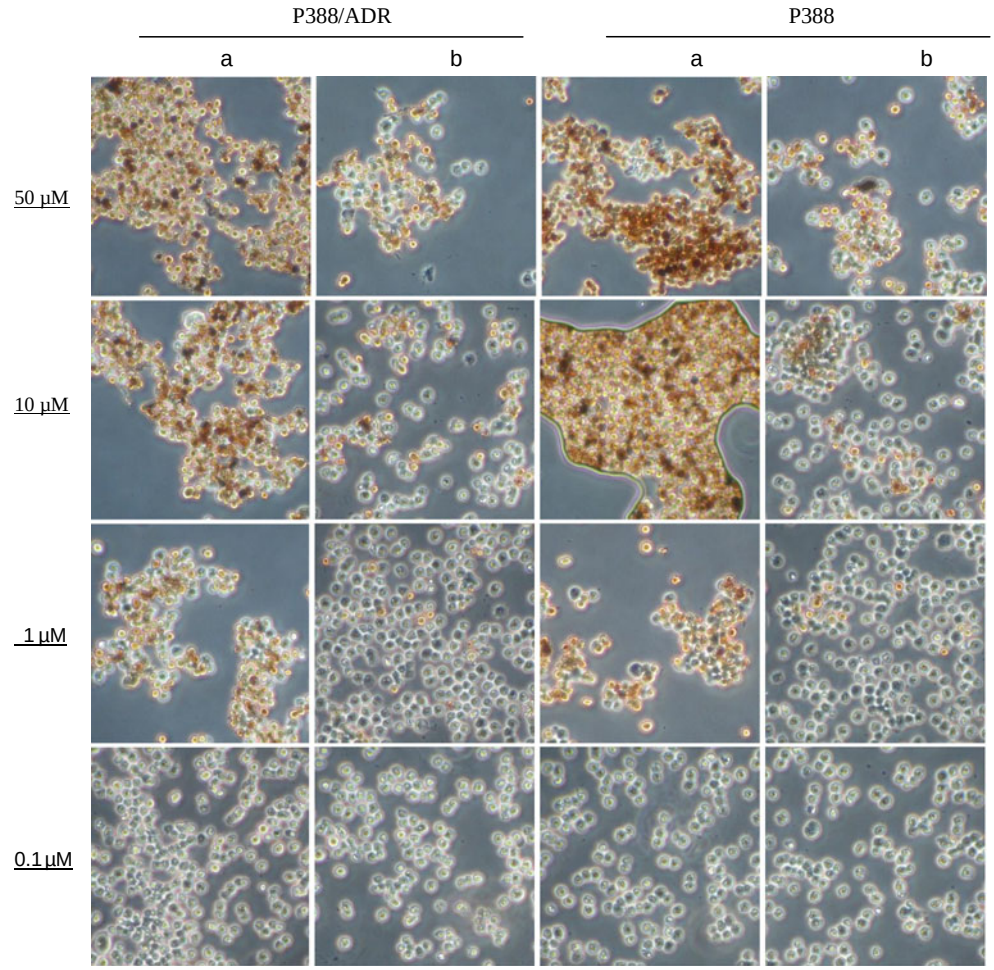

Fig. 9 Apoptosis in P388 and P388/ADR cells induced by the blank carriers at different concentrations in 10%-serum medium. The cells were treated with the blank PPC micelles (**a**) for 16 h at 37°C, 5% CO_2. The blank PP micelles (**b**) were used as a control. The DeadEnd Colorimetric TUNEL System marks the fragmented DNA *in situ* with biotinylated nucleotides by terminal transferase rTdT. The complex was detected with streptavidin-horseradish peroxidase (HRP) using the chromogen substrate diaminobenzidine and H_2O_2. As a result of the procedure the nuclei are stained dark brown. The images are representatives of at least three independent experiments. The components and their molar ratios of the blank PPC micelle and PP micelle refer to the Notes of Table I.

group at the C-1 position (N-Hexanoyl-D-erythro-sphingosine, Fig. 1d) (10). It is extremely hydrophobic by nature, potentially causing it to precipitate when its solution in DMSO or ethanol vehicle is added to cell culture media or water (15). Therefore, although it is a potent antitumor bioactive lipid, there is a critical need for improved delivery systems to maximize intracellular ceramide accumulation upon systemic administration. It was reported that C6-ceramide have been incorporated into the PEGylated nanoliposome and polymeric nanoparticles, and they have been shown to selectively induce apoptosis for a panel of tumors *in vitro* and *in vivo* (11,16–19). In this study, we used a novel PEGylated PazPC-based micellar system to co-deliver C6-ceramide and DOX. PazPC used here is an oxidized phosphocholine, containing 16-carbon, saturated fatty acyl chain (palmitic acid) at the sn-1 position and oxidatively truncated 9-carbon fatty acyl chain (azelaic acid) at the sn-2 position (Fig. 1a) (20). Recently, the DOX-loaded PazPC micelles was fabricated via electrostatic and hydrophobic interaction between PazPC and DOX by thin film-hydration process at pH7.0 in our group (6). As bioactive lipids, C6-ceramide and PazPC actually can be considered as the carrier components of a drug delivery system. In this study, PEGylated bioactive lipids-based PPC/DOX micelles, containing DSPE-PEG2000, C6-ceramide and PazPC, were prepared with the same method proposed for preparation of the DOX-loaded PazPC micelles as previously reported (6).

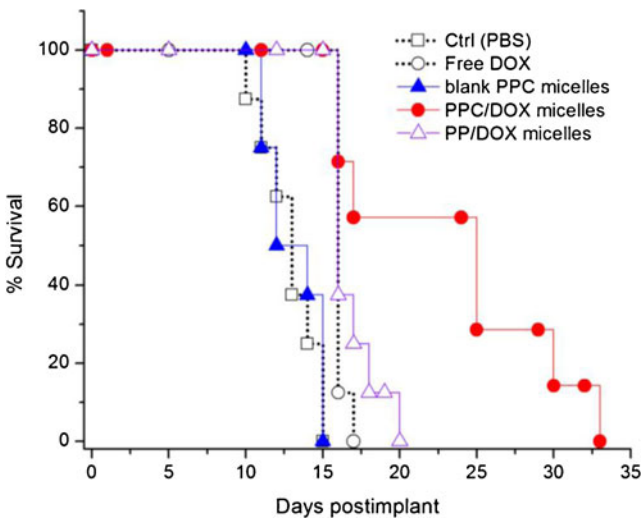

Fig. 10 Anti-leukemia efficacy of bioactive micellar DOX in CD2F1 mice bearing P388/ADR leukemia. Survival curves were derived from the different groups (8 mice/group) in male CD2F1 mice inoculated i.p. with 1×10^5 P388/ADR cells and treated 24 h later at the dose of 3.0 mg DOX/kg/injection on the q4d × 3 treatment schedules. This treatment resulted in the median survival time (mST) of 25 days and the increase in life-span (ILS) of 92% for the PPC/DOX micelles, 16.6 days and 28% for the PP/DOX micelles, 16 days and 23% for free DOX, 12 days and −7% for blank PPC micelles, respectively. The mST of PBS control was 13 days. The components and their molar ratios of the PPC/DOX micelle, the PP/DOX micelles, and the blank PPC micelles refer to the Notes of Table I.

In term of DOX encapsulation efficiency, as high as 93% DOX was encapsulated in the PPC/DOX micelles compared to 91% for the PP/DOX micelles and 63% for PazPC/DOX. This indicated that DSPE-PEG2000 and C6-ceramide provided extra hydrophobic interaction to DOX-PazPC ion-pair in the micelles and it plays an important role in stabilization of DOX in the core of the micelles. The good stability of the PPC/DOX micelles in PBS solution confirmed this type of stability (Fig. 3e–f). However, similar to PazPC/DOX micelles, because electrostatic interaction between PazPC and DOX is another driven force that maintains the micelle stability, this mixed PPC/DOX micellar system is still pH-sensitive. DOX was dissociated and released from the PPC micelles when pH was decreased from 7.4 to 5.0 (Fig. 3c–d). This pH-sensitive behavior is further confirmed by the data on cumulative release curves at different pHs, where the cumulative fraction of DOX release increased with decreasing pH in the release buffer (Fig. 4).

The antitumor activity of this bioactive lipid-based micellar DOX formulation was subsequently evaluated in murine-derived P388 and its resistant subline P388/ADR leukemia model. In *in vitro* study, compared to free DOX, free C6-ceramide and the blank PPC micelles, co-delivery of C6-ceramide and DOX in the PPC/DOX micelles resulted in IC_{50} value 7–21 folds lower in P388/ADR cell line and 16–60 folds lower in P388 cell line, and compared to the PP/DOX micelles, about 2-fold lower IC_{50} was observed in both cells, whereas the blank PP micelles didn't show any obvious cytotoxicity on both cells with the equivalent concentration of DOX-loaded micelles (Fig. 5). The *in vivo* efficacy of the PPC/DOX micelles was further confirmed in the resistant P388/ADR murine leukemia model. Compared to free DOX, blank PPC micelles and the PP/DOX micelles, the treatment with the PPC/DOX micelles resulted in significant higher median survival time (mST) of male CD2F1 mice bearing P388/ADR leukemia (Fig. 10). Interestingly, there was no survival difference between PBS control and the blank PPC micelles with the equivalent C6-ceramide and PazPC concentrations of the PPC/DOX micelles in this *in vivo* animal study. This indicates that delivery of bioactive C6-ceramide and PazPC at the current dose didn't produce observable anticancer effect, suggesting both lipids as an 'adjuvant' role in the treatment.

Further studies showed that at least three intracellular events involved in the 'adjuvant' effects of bioactive lipids on DOX activity. First, potent inhibition of P-gp efflux by bioactive lipids, especially C6-ceramide, led to a significant enhancement of intracellular uptake and retention of DOX and Rho123. The uptake of DOX was significantly increased by the PPC/DOX micelles compared to free DOX and the PazPC/DOX micelles in P388 cells and P388/ADR resistant cells during 2 h incubation (Fig. 7). Actually, the DOX uptake in resistant P388/ADR cells treated with the

PPC/DOX micelles was very close to the free DOX in sensitive P388 cells. These data suggest that the bioactive PPC micelles protected DOX from the cellular drug resistant system. In order to avoid the possible quenching DOX fluorescence during its intercalation with DNA, the experiment was repeated with Rho123, which is commonly used as a fluorescent probe for transport studies in multidrug-resistant cells and it accumulates in cells without any substantial loss of its fluorescence (21). At the concentration of 50 μM, the blank PPC micelles significantly enhanced 8.3-fold accumulation of Rho123 in P388/ADR cells, which higher than 50 μM verapamil (6.0-fold), whereas at the same concentration, the blank PP micelles only increased 2.2-fold uptake of Rho123. Interestingly, from the data of DOX uptake and Rho123 accumulation and efflux in P388/ADR cells, the PPC micelles, containing C6-ceramide, inhibited more potently against P-gp than the PP micelles without C6-ceramide, indicating that C6-ceramide played a major role to overcome drug resistance in P388/ADR cells. The inhibition of P-gp function is possible related to the mitochondria damage induced by bioactive lipids. It was reported that C6-ceramide decreases mitochondrial membrane permeabilization (MMP) (7,8,22,23), which possibly depresses the production of ATP and subsequently affects the function of ATP-dependent MDR-related protein, such as P-gp, resulting in increasing intracellular drug. Another possible mechanism of inhibitory P-gp is from the perturbed lipid bilayer of cells. Both bioactive lipids used in this study, C6-ceramide and PazPC, having 2 asymmetric hydrophobic chains, readily destroy or perturb membrane integrity during the micelles are uptaken by the cells, in which P-gp exert its function depend on the properties of the lipid bilayer (24,25). Second, the enhanced antitumor effects of bioactive lipids were from intensified apoptosis induced by C6-ceramide and PazPC. It was previously reported that PazPC and C6-cermaide can associate with mitochondria, resulting in cytochrome c releasing from mitochondria into the cytoplasm and nucleus (7,8,22,23) to induce apoptosis in variety of tumor cells (7,9), but the former only induce slightly reversible apoptotic effect and the latter is more potent proapoptotic agent. This was confirmed by our data of apoptotic effect of the blank PPC micelles and the blank PP micelles at different concentrations in P388/ADR and P388 cells (Fig. 9). Notably, DOX induces apoptosis in tumor cells partially through generating ceramides intracellularly. In some resistant tumor cells, however, DOX- induced ceramides are metabolized to non-toxic species by glucosylceramide synthase (GCS) (11,12). Therefore, our strategy to apply exogenous C6-ceramide into the resistant cells may inhibit GCS activity through substrate inhibition to re-establish DOX-induced cell apoptosis and may directly induce apoptosis. Presumably, the synergistic effect between DOX and C6-ceramide, as well as the enhanced antitumor efficacy of the PPC/DOX micelles *in vitro* and *in vivo* are attributable to this re-established apoptosis in the tumor cells. Third, completely different intracellular distribution of DOX and C6-ceramide after endocytosis is probably a mechanism that led to synergistic effect between DOX and C6-cermaide. In the beginning of uptake of the PPC/DOX micelles by P388/ADR cells, C6-ceramide and DOX had similar intracellular distribution (cytoplasm); at the late stage, majority of DOX was escaped from pH sensitive micelles in acidic endosome/lysosome compartments and located in the nucleus in which DOX intercalates into DNA, blocking topoisomerase II activity, preventing DNA replication and cell division, whereas C6-ceramide distribution was still located in the cytoplasm (Fig. 6).

In summary, our studies provide evidence for the significant enhancement of DOX efficacy by bioactive lipids-based micelles *in vitro* and *in vivo* in a resistant leukemia model. Current results also indicate that the inhibition of P-gp function by bioactive lipids, intensified apoptosis induced by bioactive lipids and delivery of DOX into nucleus by pH sensitive micelles could explain the enhanced efficacy in resistant leukemia both *in vitro* and *in vivo*. This bioactive lipids-based micellar system with C6-ceramide and PazPC as major components can therefore be developed to deliver chemotherapy drugs for resistant leukemia treatment in the future.

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