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Oxidized phospholipid based pH sensitive micelles for delivery of anthracyclines to resistant leukemia cells *in vitro*

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ABSTRACT

A self-assembled micelle drug delivery system was constructed with an oxidized phospholipid for anthracycline anti-cancer drug delivery. An oxidized phospholipid, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazPC), was chosen to fabricate micelles via both electrostatic and hydrophobic interactions for delivery of doxorubicin (DOX) and idarubicin (IDA). The formation of ion–pair complexes between PazPC and DOX was first investigated under different pH conditions. Drug-loaded PazPC micelles at a 5:1 molar ratio of lipid/drug at pH 7.0 were then prepared by the solvent evaporation method. The empty and drug-loaded PazPC micelles exhibited a small particle size (~10 nm) and high encapsulation efficiency. *In vitro* stability and release profile indicated that the micelles were stable at physiological conditions, but exhibited pH-sensitive behavior with accelerated release of DOX or IDA in an acidic endosome environment. Finally, *in vitro* uptake and cytotoxicity were evaluated for leukemia P388 and its resistant subline P388/ADR. The drug-loaded PazPC micelles enhanced drug uptake and exhibited higher cytotoxicity in both leukemia cells in comparison to free drugs. In conclusion, we developed a novel pH sensitive oxidized phospholipid-based micellar formulation which could potentially be useful in delivering anthracycline anti-cancer drugs and provide a novel strategy for increasing the therapeutic index while overcoming multidrug resistance for leukemia treatment.

1. Introduction

Self-assembled micelles from amphiphilic molecules have attracted considerable interest and have been comprehensively studied due to their unique advantage as a drug delivery system for anticancer drugs (Kim et al., 2009; Liu et al., 2010; Tyrrell et al., 2010). The benefits of micellar formulations include their small size (10–100 nm), core–shell structure which leads to increased solubility and metabolic stability of the associated drugs, and passive or active targeting capability which enhances the specificity of drug activity (Tyrrell et al., 2010). The core of a micellar system serves as a micro-reservoir that incorporates drug molecules through a combination of hydrophobic and electrostatic interaction, hydrogen bonding, and, in some cases, chemical conjugation (Kim et al., 2009; Tyrrell et al., 2010). Among the micellar drug delivery systems, many amphiphilic polymers, including block, graft and linear-dendritic polymer, have been widely used to fabricate micelles to improve drug efficacy and therapeutic index. For instance, a poloxamer-based micellar formulation of doxorubicin (DOX), SP1049C, was shown to be safe and effective in animals and humans (Alakhov et al., 1999; Danson et al., 2004).

Among amphiphilic polymers, lipid derivative polymers have been widely investigated. For instance, some phospholipid derivatives, e.g., DSPE-PEG and DOPE-PEG, have demonstrated many advantages for delivery of hydrophobic drugs (Lukyanov and Torchilin, 2004; Koo et al., 2005). Phospholipids represent a well-known class of biocompatible and non-cytotoxic amphiphilic biomolecules. Phospholipids have a hydrophilic phosphate ester 'headgroup' and two aliphatic chains as hydrophobic 'tails'. They typically form bilayer vesicles, i.e., liposomes, rather than a micellar structure. However, some lipid derivatives can form micelles when their headgroups are modified with hydrophilic polymers, e.g., PEG or PVP, etc. (Lukyanov and Torchilin, 2004). In addition, when one of the two hydrophobic chains at the glycerol backbone is oxidatively truncated (i.e., oxidized phospholipids), this class of phospholipid derivatives also spontaneously forms micelles in water (Mattila et al., 2007; Vitiello et al., 2009). Oxidized phospholipids (OxPLs) are metabolic products of phosphatidylcholine produced through tissue damage, oxidative stress, or inflammatory stimulation. They are components of cell membranes and circulating lipid particles, and constitute an important subclass of phospholipids that exhibit unique physical and biological properties not found in their parent phospholipids. As endogenous phospholipid derivatives, their biological activities are widely characterized, including anti- and pro-inflammatory effects, apoptotic effects, and potent ligands for scavenger receptors (Fruhwirth et al., 2007; Bochkov et al., 2010).

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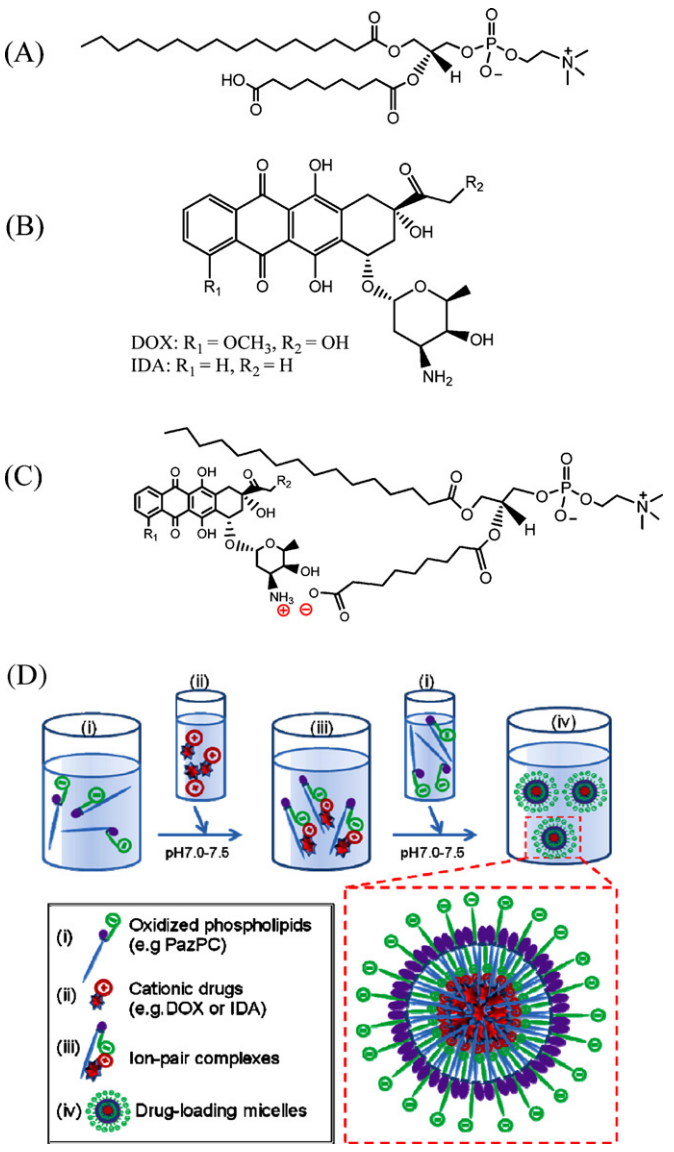

Fig. 1. The structure of oxidized lipid, PazPC (A) and the cationic drugs, doxorubicin (DOX) and idarubicin (IDA) (B), ion–pair complexes between PazPC and DOX or IDA (C) and self-assembly of drug-loaded micelle (D).

However, only a few papers have discussed the physicochemical properties of the micellar aggregates of oxidized phospholipids *in vitro* (Mattila et al., 2007; Pande et al., 2010). Furthermore, no work has described the micellar system formed by these phospholipid derivatives for drug delivery *in vitro* and *in vivo*.

Due to the unique features of their structural organization and biological activity, oxidized phospholipids can be used to construct drug delivery systems. In this study, we examined the possible use of oxidized phospholipid-based micelles for delivery of anthracyclines. An oxidized phospholipid, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazPC, Fig. 1A), was chosen as a model compound. PazPC is a truncated oxidized phosphotidylcholine with a 9-carbon azelaoyl chain at the sn-2 position, and a 16-carbon palmitoyl chain at the sn-1 position, produced from the oxidation of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC) (Pande et al., 2010). It was predicted that the polar carboxyl group of the truncated sn-2 acyl chain reverses its orientation and reaches the lipid–water interface when micellar aggregates are spontaneously formed at neutral pH in water (Pande et al., 2010; Khandelia et al., 2009). We hypothesize that the negatively charged carboxyl group of the azelaoyl chain at the sn-2 position could form an ion–pair (Fig. 1C) with the primary amine of anthracyclines, i.e., DOX and IDA (Fig. 1B). At the same time, the hydrophobic interaction between aromatic rings of the drugs with the sn-1 chain of the lipid should further stabilize the micelles. Therefore, once the micelle is formed, the drugs will be effectively encapsulated in the core of the micelles (Fig. 1D).

In the present study, the conditions of ion–pair complex formation and subsequent micelle preparation were optimized. Characterization of the OxPLs-based micellar delivery system and *in vitro* drug release studies under different pH values were performed. Finally, uptake and cytotoxicity of the micellar drug formulations in sensitive and drug resistance leukemia cell lines were tested.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Doxorubicin hydrochloride (DOX), idarubicin hydrochloride (IDA), HEPES, Sephadex G75 and PBS were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum, RPMI1640 medium and other reagents for cell culture were purchased from Mediatech (Manassas, VA). Murine leukemia cell lines, P388 and P388/ADR, were obtained from the National Cancer Institute at Frederick (Frederick, MD).

2.2. Formation of PazPC/DOX ion–pair complexes

DOX solutions (3 mM) in 20 mM HEPES buffer with different pH values of 4.5–9.0 were freshly prepared. PazPC in chloroform were formed as films in glass tubes under nitrogen and desiccated under vacuum. A predetermined volume of the DOX solutions were added to the lipid film-containing tubes, and hydrated at 60 °C for 30 min to obtain a 1:1 molar ratio of PazPC/DOX. Scattering intensities of the hydration mixtures were determined using a Nicomp 380 Submicron Particle Sizer (PSS, Santa Barbara, CA). The mixtures were centrifuged for 10 min at 10,000 rpm. The concentrations of DOX in the supernatant of the ion–pair complex were measured fluorometrically at 480 nm (excitation) and 590 nm (emission). The percentage of the drug ion-paired with PazPC (mol/mol) was calculated as follows (Ma et al., 2009):

$$\%\text{ of the drug ion-paired with PazPC} = 100\% - (\%\text{ of drug in supernatant}) \quad (1)$$

In order to eliminate the influence of the DOX solubility, control experiments used DOX solutions without PazPC (pH 4.5–9.0) and the % precipitates formed were subtracted to obtain the net % of DOX ion-paired with PazPC.

2.3. Preparation of PazPC/DOX or IDA micelles

100 μl PazPC (10 mg/ml) in chloroform was pipetted into a glass tube, the solvent was removed under a stream of nitrogen, and the lipid films were maintained under vacuum for at least 2 h. The dry lipid films were hydrated at 60 °C for 30 min in 1 ml DOX or IDA (0.3 mM) in 20 mM HEPES buffer (pH 7.0) to yield a 5:1 molar ratio of PazPC/drug. The drug-containing PazPC micelle solution was placed in a bath-type sonicator for 10 min. The prepared PazPC/DOX and PazPC/IDA micelles were used in subsequent experiments without further purification.

2.4. Characterization of PazPC/DOX or IDA micelles

2.4.1. Encapsulation efficiency and drug loading content

The ratios of DOX or IDA encapsulated in PazPC micelles were determined using a column separation method. Sephadex G75 was employed to separate the PazPC/DOX or PazPC/IDA micelle solution from free drug. A 0.5 ml micelle sample was added to the column and eluted using 20 mM HEPES buffer (pH 7.0) and 1.5 ml fractions were collected. The fluorescence intensity of DOX in each tube was measured at 480 nm (excitation) and 590 nm (emission) using a microplate reader (Fluostar, BMG Labtechnologies, Germany). Elution profiles of DOX or IDA were then plotted versus elution volumes. The first peak (fractions 3–6) reflects the micellar drug and the second peak (fractions 7–16) reflects free drug. The fractions of each peak were combined and the concentration of DOX or IDA was quantified fluorometrically at 480 nm (excitation) and 590 nm (emission). The encapsulation efficiency (EE%) and drug loading content (DL%) of PazPC micelles were calculated as follows (Wang et al., 2007):

$$\text{Encapsulation efficiency}\,(\%) = \frac{\text{amount of micellar drug}}{\text{total amount of drug}} \times 100\% \quad (2)$$

$$\text{Drug loading}(\%) = \frac{\text{amount of micellar drug}}{\text{total amount of lipid} + \text{amount of micellar drug}} \times 100\% \quad (3)$$

2.4.2. Particle size and zeta potential characterization

The particle size and zeta potential of the PazPC micelles were determined from dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively, using a Nicomp Model 380/ZLS particle sizer (PSS, Santa Barbara, CA).

2.4.3. Stability in acidic pH, PBS, and serum

The stability of drug-loaded PazPC micelles was evaluated under different conditions. The PazPC/DOX and PazPC/IDA micelles were prepared at pH 7.0. In order to evaluate micelle stability at a lower pH, the pH of the sample was adjusted to 6.0 using 0.6 M HCl, incubated at 37 °C for 30 min, applied to a Sephadex G75 column, and eluted with 20 mM HEPES buffer at pH 6.0. In order to evaluate stability at a physiological ionic strength or in normal cell culture medium, aliquots of 25× PBS or fetal bovine serum (FBS) were added to the micelle solution to yield a final concentration of 1× PBS or 10% serum, respectively. Two samples were incubated at 37 °C for 30 min, applied to a Sephadex G75 column, and eluted with 1× PBS-containing 20 mM HEPES buffer or 10% FBS-containing 20 mM HEPES buffer, respectively. Elution profiles of DOX or IDA (λ_{ex} = 480 nm and λ_{em} = 590 nm) were plotted versus elution volumes.

2.4.4. In vitro release property

DOX or IDA release from PazPC micelles was studied at different pH values. One ml of PazPC/DOX or PazPC/IDA or free drug solutions (0.3 mM DOX or IDA in each sample) was placed in a dialysis membrane (MWCO 15 K, Spectra/por membrane tubing, Spectrum Labs, CA) and immersed in tubes containing 40 ml of release buffers at different pH values (1× PBS, pH 7.4 or pH 6.5; 0.1 M citrate buffer, pH 6.0). All tubes were incubated at 37 °C under mild agitation. The dialysate sample (0.5 ml) was collected at different time intervals and replenished immediately with the same volume of the fresh medium. The concentration of DOX or IDA in the dialysate was analyzed fluorometrically at 480 nm (excitation) and 590 nm (emission), and cumulative release profiles were plotted verse release times.

2.5. Uptake of DOX and IDA micelles by leukemia cells

The cellular uptake of PazPC micellar drug and free drug was first visualized by a fluorescent microscope (EVOS fl Microscope, AMG, USA). Briefly, P388 and P388/ADR cells were seeded into 12-well plates at a cell density of 10^6 cells/ml and PazPC micellar drug or free drug (DOX: 10 μM, IDA: 1 μM) was added. After 2 h of incubation, the cells were fixed (4% paraformaldehyde, Biolegend) for 20 min at room temperature. Cellular uptake of micellar drug or free drug was finally visualized under the light channel of red florescence.

The extent of cellular uptake of PazPC micellar drug or free drug was further quantitated using a flow cytometry method (Chen et al., 2010) (Accuri C6 Cytometer, Ann Arbor, MI) by measurement of the cell associated fluorescence. P388 and P388/ADR cells were seeded on 12-well plates at a cell density of 10^6 cells/ml. The appropriate concentration of each formulation or free drug (DOX: 10 μM, IDA: 1 μM) was added to the cells and incubated at 37 °C for different times. The fluorescence was then measured by flow cytometry by collecting 20,000 events for each sample at predetermined times. Each experiment was performed in triplicate.

2.6. Cytotoxicity of leukemia cells

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was utilized to assess the cytotoxicity of the PazPC micelles. Cells were seeded in 96-well plates at 5000 cells in 100 μl RPMI1640 medium with 10% FBS per well. Serial dilutions of DOX or IDA formulations were added to the plate with 10% FBS at 37 °C in 5% CO_2 for 48 h. Ten 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 centrifuged for 10 min at 1000 rpm. The medium was removed and 100 μl DMSO was added to each well to solubilize the dye. The absorbance was measured using a 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" (OriginPro 8.0).

2.7. Statistical analysis

The data were expressed as mean ± S.D. Statistical significance in uptake and cytotoxicity were determined using one-way ANOVA followed by a Student's t test for multiple comparison tests. A p value of <0.05 was considered as statistically significant.

3. Results

In order to obtain an optimal pH for formation of an ion–pair complex between oxidized phospholipid and DOX, the changes in the scattering intensity and the resultant precipitates of mixtures of PazPC and DOX (molar ratio = 1:1) as a function of pH were investigated. As shown in Fig. 2A, the scattering intensities reached a maximum at pH 7.0–7.5, and the intensity decreased at pH <7.0 or >7.5, as indicated by the bell-shaped curve. Similar results were obtained by centrifugation of the mixtures of PazPC and DOX. The percentage of the drug ion-paired with PazPC was greater than 60% at pH 7.0 and 7.5, but it dramatically decreased at pH <7.0 or >7.5. Therefore, pH 7.0–7.5 is the optimal pH range to form the PazPC/DOX ion–pair complexes. In order to optimize the ratio of lipid to drug for preparation of drug-loaded micelles with reasonable particle size and encapsulation efficiency, DOX concentration was kept constant (0.3 mM) and different concentrations of PazPC

Table 1
Characterization of PazPC/DOX micelles and PazPc/IDA micelles (PazPc/drug = 5:1, molar ratio).

	Particle size (nm)	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (%)
Blank PazPC micelle	7.7 ± 0.9	−39.2 ± 5.3	–	–
PazPC/DOX mcielle	8.5 ± 1.0	−27.8 ± 2.5	62.5	10.4
PazPC/IDA micelle	8.9 ± 0.9	−24.3 ± 3.7	87.1	14.8

were used to prepare PazPC/DOX micelles. The changes in particle size, scattering intensity, encapsulation efficiency, and drug loading content of PazPC/DOX micelles as a function of PazPC/DOX molar ratio is shown in Fig. 2B and C. When the ratio of PazPC/DOX was greater than 2:1, the intensity of the PazPC/DOX mixtures dramatically decreased to around 25 kHz compared to 170 kHz (1:1 ratio) or 260 kHz (1:2 or 1:10 ratio). The particle size showed similar changes when the ratio was greater than 2:1 (Fig. 2B). This indicated that the DOX-loaded PazPC micelle was formed through ion–pair interaction between PazPC and DOX when the ratio was greater than 2:1. Fig. 2C also shows that encapsulation efficiency (%) increased and drug loading (%) decreased with increasing of the ratio, respectively. When the ratio was greater than 5:1, the encapsulation efficiency (%) was greater than 60%, and drug loading (%) was around 10%. With the ratio of 7:1, the encapsulation efficiency (%) increased to 80%, but the drug loading (%) decreased to less than 10%. Fig. 2D represents intensity-weighted size distribution of the PazPC/DOX micelles with a molar ratio of 5:1. Blank PazPC micelles and PazPC/IDA micelles has a similar size distribution by DLS (data not shown). The molar ratio of 5:1 (PazPC:DOX or IDA) was then chosen for subsequent experiments.

The characterization of PazPC/DOX and PazPC/IDA micelles prepared at a 5:1 molar ratio of lipid/drug is summarized in Table 1. The particle size of blank PazPC micelles, PazPC/DOX micelles and PazPC/IDA micelles, measured by DLS, was in the range of 8–10 nm. Table 1 also shows that the PazPC/IDA micelles had a comparable zeta potential and higher encapsulation efficiency (%) compared to PazPC/DOX micelles. More IDA was entrapped in the core of PazPC micelles than DOX, presumably due to higher hydrophobicity of IDA. However, both PazPC/DOX and PazPC/IDA micellar formulations contained free drug in the preparation. The data also indicated that both electrostatic and hydrophobic interactions were involved in the driving force of micelle formation.

Next, the stability of PazPC/drug micelles was studied using G75 size exclusion chromatography. Fig. 3 shows the stability of drug-loaded PazPC micelles under different conditions: acidic pH, neutral physiological salt concentration and 10% serum. As shown in Fig. 3A and B, DOX or IDA significantly dissociated from the PazPC micelle when the micelles were incubated at pH 6.0 for 30 min, resulting in the area of the first peak dramatically decreasing and the second peak increasing. When the micelles were incubated in 1× PBS buffer for 30 min, the second peak of PazPC/DOX or PazPC/IDA micelles increased just slightly (Fig. 3C and D), and encapsulation efficiency of the micelles was maintained at over 60% (for DOX) or 80% (for IDA) (Table 2). When the micelles were incubated in 10% fetal bovine serum for 30 min, the changes in elution profiles of the micelles were similar to that in 1× PBS buffer (Fig. 3E and F).

The pH-dependent release profiles of DOX or IDA from the drug-loaded PazPC micelles are shown in Fig. 4. A control experiment

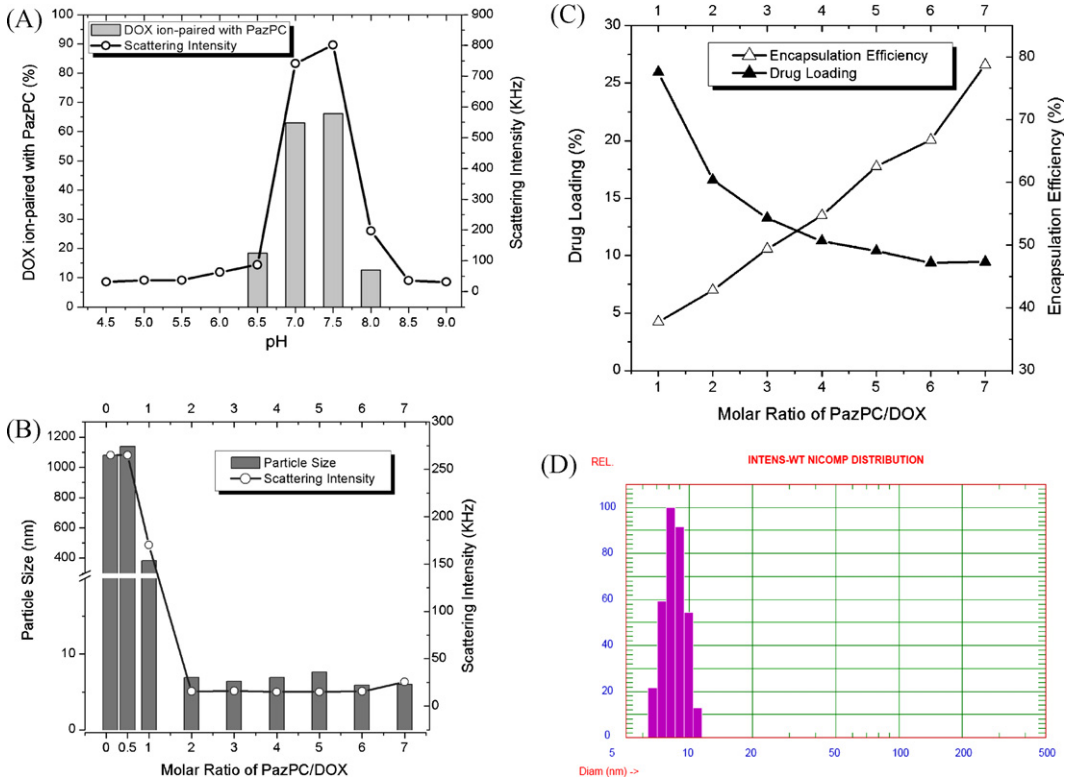

Fig. 2. Formation and characterization of PazPC/DOX ion–pair complex and micelles. (A) The effect of pH on the formation of PazPC/DOX ion–pair complexes. The molar ratios were 1:1. (B) The effect of the PazPC/DOX molar ratio on the particle size of DOX-loaded PazPC micelle. (C) The effect of the PazPC/DOX molar ratio on encapsulation efficiency and drug loading content of DOX-loaded PazPC micelles. (D) Representative Nicomp distribution analysis (intensity-weighted) of the PazPC/DOX micelles (8.5 ± 1.0 nm) by DSL. All measurements were done in triplicate.

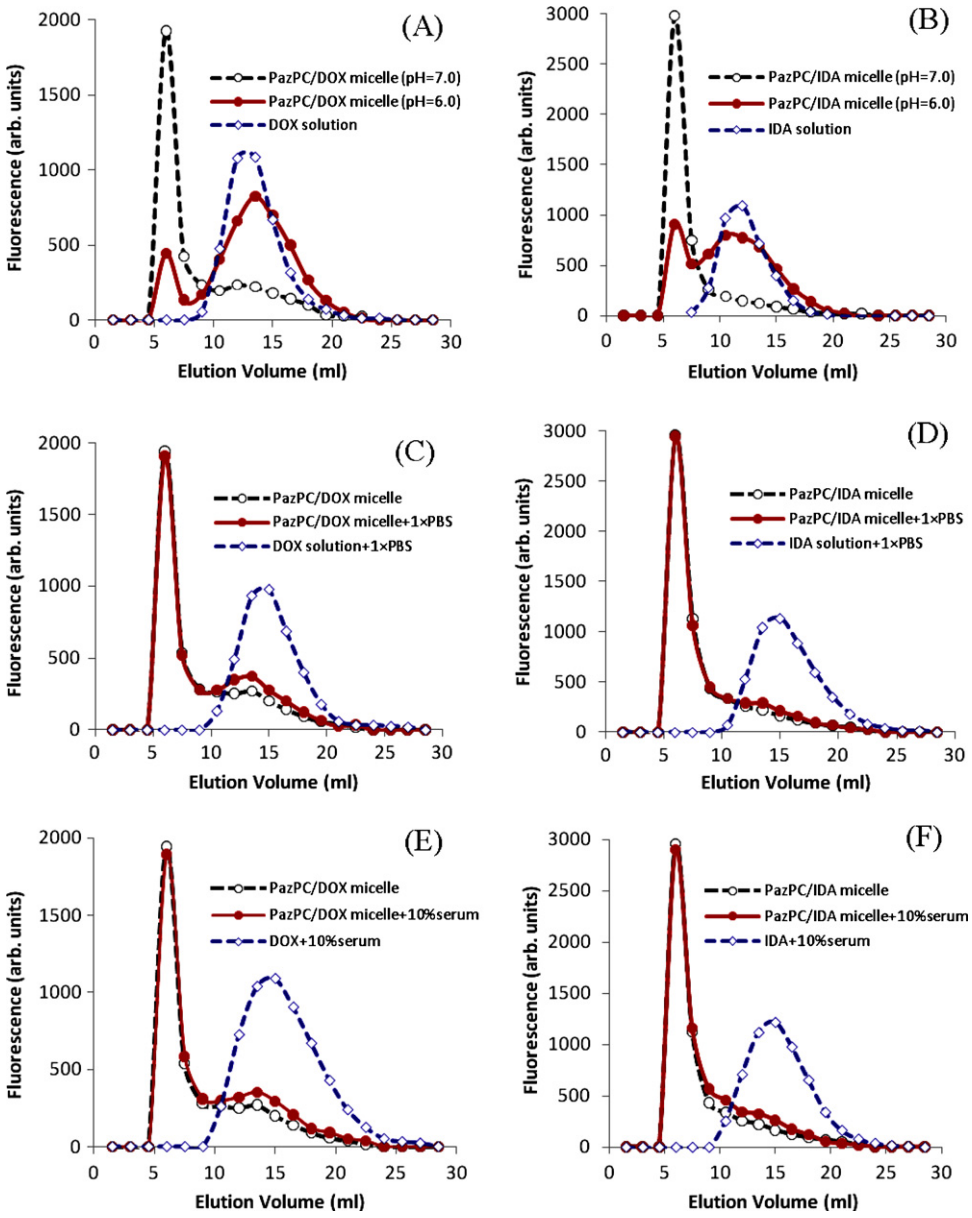

Fig. 3. *In vitro* stability of PazPC/DOX and PazPC/IDA micelles (PazPC/drug = 5:1, molar ratio) under different conditions: acidic pH (A and B), 1× PBS (C and D) and 10% serum (E and F).

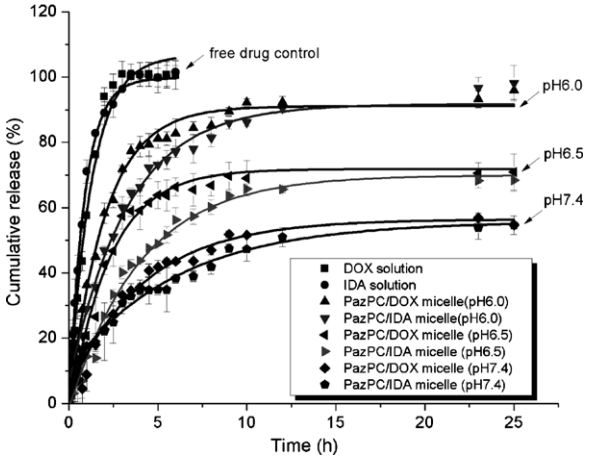

Fig. 4. *In vitro* release profiles of DOX and IDA from drug-loaded PazPC micelles (PazPC/drug = 5:1, molar ratio) at different pH.

with free DOX and IDA confirmed that the high MWCO (15 K) dialysis membrane tubing could not restrict diffusion of the released free drug, and reached 100% release after 3.5 h. However, the release of DOX or IDA from the micelles at different pH values could not reach a plateau for at least 10 h. The total released drug from the micelles was significantly different under different pH conditions. The release rate of drug from PazPC micelles increased with decreasing pH of the release medium. Interestingly, at any

Table 2
Encapsulation efficiency (EE%) of PazPC/DOX and PazPC/IDA micelles under different conditions.

	pH = 7.0			pH = 6.0
	–[b]	1× PBS	10% serum	
PazPC/DOX micelle[a]	61.9	61.0	58.2	4.8
PazPC/IDA micelle[a]	86.6	84.7	81.9	14.4

[a] PazPC/drug = 5:1, molar ratio, and [drug] = 0.3 mM.
[b] Normal drug-loaded PazPC micelles, without 1× PBS or 10% serum.

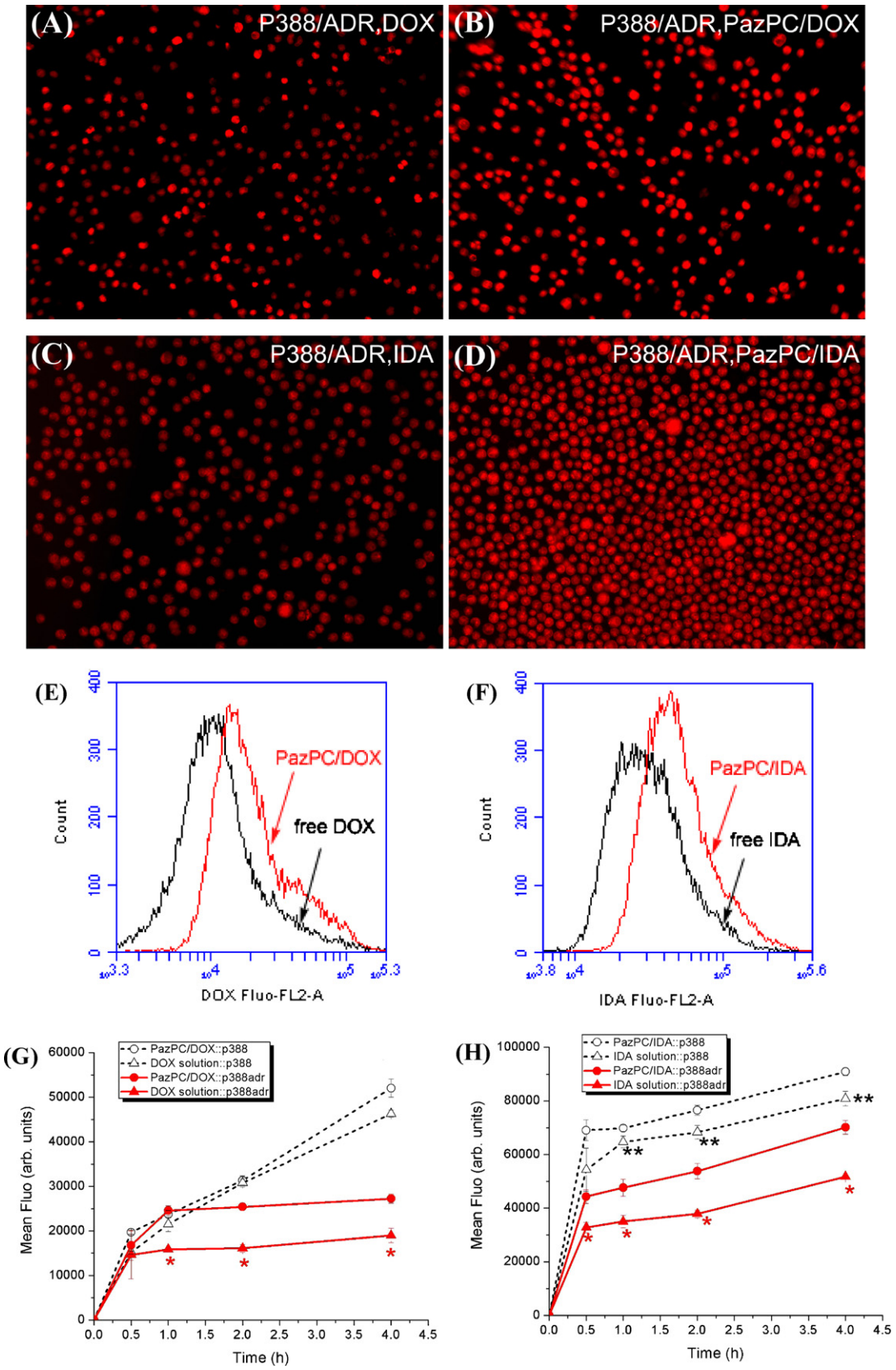

Fig. 5. Cellular uptake of drug-loaded PazPC micelles. (A–D) Cellular uptake visualized by fluorescence microscope (20×) through treating cells for 2 h in a leukemia resistant cell line p388/ADR, (A) free DOX, (B) PazPC/DOX micelles, (C) Free IDA, (D) PazPC/IDA micelles; (E and F) cellular uptake analysis by flow cytometry through treating cells for 2 h, (E) free DOX and PazPC DOX micelles, (F) free IDA and PazPC IDA micelles. (G and H) The time courses of PazPC micellar DOX or IDA uptake by flow cytometric analysis in leukemia cells treated with drug-loaded PazPC micelles (PazPC/drug = 5:1, molar ratio): (G) DOX for 0–4 h, (H) IDA for 0–4 h. $^{*}p < 0.01$, and $^{**}p < 0.05$, as compared with free drug.

Table 3
IC_{50} values (nM) of free DOX, IDA and their PazPC micelles (PazPC/drug = 5:1, molar ratio) on leukemia cells (48 h).

	P388	P388/ADR
Free DOX	221.20 ± 63.31	2424.75 ± 289.94
PazPC/DOX	26.34 ± 2.95*	166.07 ± 49.83*
Free IDA	59.88 ± 15.61	361.16 ± 5.05
PazPC/IDA	10.93 ± 2.75*	45.11 ± 9.97*
Blank PazPC (μM[a])	93.34 ± 2.55	136.90 ± 1.29

* $P < 0.01$, as compared with free drug.
[a] The unit of IC_{50} value for the blank PazPC micelles is μM.

given pH, the release of IDA from the micelles displayed a slightly slower rate than DOX before the plateau was reached.

The cellular uptake of DOX and IDA with micellar formulations was examined in sensitive P388 and resistant P388/ADR leukemia cell lines using fluorescence microscopy. After 2 h of incubation, an enhanced uptake of PazPC/DOX micelles was observed in resistant leukemia cell line P388/ADR compared to free DOX (Fig. 5A and B). As for PazPC/IDA micelles, an enhanced uptake was observed in the resistance leukemia cell line compared to free IDA (Fig. 5C and D). The uptake of DOX or IDA by leukemia cells was then quantitatively investigated using a flow cytometry by measurement of cell-associated fluorescence intensity (Chen et al., 2010). Fig. 5E and F shows that the cellular uptake of DOX or IDA was significantly increased by the micelle formulations compared to free drug in P388/ADR resistant cells after a period of 2 h of incubation. Fig. 5G and H present the 4 h time courses of drug uptake by leukemia cells treated with DOX (10 μM) or IDA (1.0 μM) micelles or free drug. DOX or IDA levels gradually increased at different rates during the 4 h treatment. In the case of DOX uptake (Fig. 5G), in sensitive P388 cells treated with free drug or micellar drug, there is no difference in DOX uptake, but in resistant P388/ADR cells, the treatment of micellar DOX increased DOX uptake compared to free DOX treatment ($p < 0.01$) after 1 h of treatment. When the cells were treated with free IDA or PazPC/IDA micelles, the micellar formulation induced higher IDA uptake in both sensitive and resistant cells than free IDA. However, in sensitive cells, the p values are less than 0.05 for all the time points after 2 h of treatment, whereas in resistant P388/ADR cells, the p values are less than 0.01 for all the time points after 0.5 h of treatment (Fig. 5H). In order to determine whether the enhancement of cellular uptake simply resulted from the effects of the lipid, the leukemia cells were treated with blank PazPC micelles and free drug by simple mixing or sequentially. When both cell lines were first incubated with blank micelles for 2 h, then washed, and treated with free drug for another 2 h, the uptake of DOX or IDA did not increase compared to that of free drug (Supplemental Fig. 1A and B). Furthermore, simple mixing of blank micelles with free drug for 2 h only slightly increased the drug uptake in sensitive and resistant cell lines (Supplemental Fig. 1A and B). In summary, the micellar drug formulations induced higher cellular drug uptake than free drug or a simple mixture of drug and empty micelles in both resistant and sensitive leukemia cells.

In vitro cytotoxicity of free DOX, IDA, PazPC/DOX, PazPC/IDA micelles, and blank PazPC micelles were conducted in sensitive P388 and resistant P388/ADR cells. The IC_{50} values are listed in Table 3, and cell viability of both leukemia cells is shown in Fig. 6. PazPC/DOX and PazPC/IDA micelles exhibited significantly higher cytotoxicity ($p < 0.01$) in both cell types compared to free drug. In the case of the resistant P388/ADR cell, the IC_{50} values of PazPC/DOX micelles were 14-fold lower than that of free DOX, and PazPC/IDA micelles were 8-fold lower than free IDA. In contrast, the IC_{50} values were 8-fold lower for DOX micelles and 5-fold lower for IDA micelles in the sensitive P388 cells. Therefore, the resistant P388/ADR cell is more susceptive to the PazPC micellar drugs than their sensitive counterparts. More importantly, the cytotoxicity

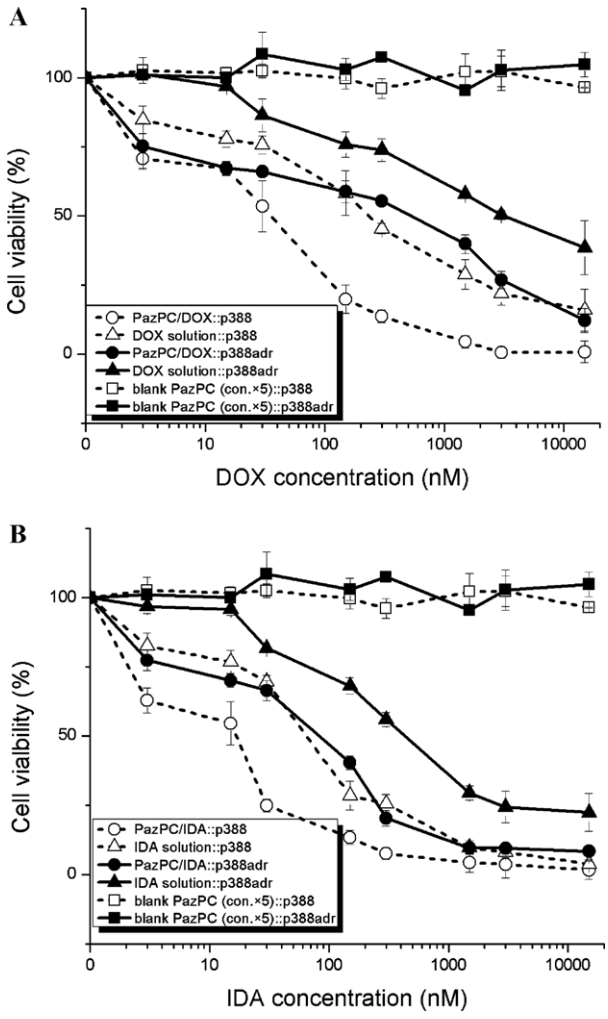

Fig. 6. Cell viability of leukemia cells treated with PazPC/DOX micelles (A) or PazPC/IDA micelles (B) (PazPC/drug = 5:1, molar ratio) for 48 h.

of PazPC micellar drug in resistant cells was comparable to the cytotoxicity of free drug in sensitive cells (Fig. 6), resulting in comparable IC_{50} values (166 nM vs 221 nM for DOX, and 45 nM vs 59 nM for IDA, Table 3). Table 3 also shows that the blank PazPC micelles had very high IC_{50} values in sensitive P388 cells (93 μM) and resistant P388/ADR cells (136 μM), and showed no cytotoxicity on either cell type with the equivalent concentration of drug-loaded PazPC micelles (Fig. 6).

4. Discussion

The CMC value of PazPC is about 18.7–23.1 μM (Mattila et al., 2007; Pande et al., 2010), which is slightly higher than the CMC of lipids currently used in micelle drug delivery system, such as DSPE-PEG2000 (CMC = 11 μM) (Lukyanov and Torchilin, 2004). Both hydrophobic and electrostatic interactions are involved in the interaction between DOX and PazPC (Mattila et al., 2007). By forming the ion–pair complex, the anionic charge of the carboxylic group of the sn-2 azelaoyl chain of PazPC is neutralized by the cationic charge of the drug. Accordingly, the hydrophobic interaction between the aromatic rings of the drug and sn-1 chain will force the sn-2 short chain to turn back and interact with the sn-1 chain. In Fig. 2B, the 1:1 molar ratio of PazPC to DOX formed approximately 400 nm size particles, which is the size range of liposomes formed by a natural phosphatidylcholine. This suggests that the complex of 1:1 molar ratio might form a structure similar to an

intact phosphotidylcholine, e.g., DPPC (dipalmitoylphsophatidylcholine). However, more experiments are needed to elucidate the exact packing arrangement of the ion–pair complex. When the negatively charged hydrophilic carboxyl group is neutralized by DOX, the CMC should be much lower than that of free PazPC. In our system, the drug-loaded PazPC micelles were prepared at a 5:1 molar ratio of PazPC to drug. Thus, our system contained excess of free PazPC in addition to the ion–pair complexes of PazPC/drug. Therefore, the resultant micellar system could be considered a mixed binary micelle system consisting of PazPC/drug ion–pair complexes and free PazPC. Accordingly, the CMC of mixed binary surfactants in aqueous solution can be calculated as (Cheng and Chen, 2005):

$$\frac{1}{CMC_{mix}} = \frac{\alpha}{CMC_A} + \frac{1-\alpha}{CMC_B} \quad (4)$$

where CMC_{mix}, CMC_A and CMC_B represent the CMC of the mixed surfactants (drug loaded and free PazPC micelles), surfactant A (PazPC/drug ion–pair complex) and surfactant B (free PazPC), respectively. Alpha (≤ 1) is the mole fraction of surfactant A in the binary system. Because CMC_A is less than CMC_B, according to Eq. (4), the CMC of drug loaded and free micelles (CMC_{mix}) should be less than the CMC_B, but greater than CMC_A ($CMC_A \leq CMC_{mix} \leq CMC_B$), suggesting that the drugs actually stabilized the micelles. In conclusion, the DOX or IDA-loaded PazPC micelles showed good stability, which resulted from the lower CMC of the PazPC/drug ion–pair complexes compared to PazPC itself, as well as two driving forces (electrostatic and hydrophobic) that govern the formation of the drug-loaded PazPC micelles.

In order to maintain drug–micelle stability, the ion–pair complex must be effectively formed between PazPC and drug. If the conditions for formation of the complex are removed, the micelle will not be stable and drug will dissociate. This was confirmed by the stability data at pH 6.0, where only about 5% DOX and 14% IDA are associated with the micelle compared to 62% DOX and 87% IDA at pH 7.0. pH-sensitive drug release from delivery systems is believed to be advantageous for tumor targeting and endosome escaping since the interstitial space of solid tumors and intracellular endosome compartments have a lower pH (Kim et al., 2009; Gao and Chan, 2010). Due to the higher hydrophobicity of IDA, the IDA-loaded PazPC micelles have a higher EE%, resulting in a greater number of IDA molecules entrapped in the core of the micelle and a lower CMC of the binary system. As a result, IDA-loaded PazPC micelles have a relative high stability against dilution.

Our data indicate that when the cells were pretreated with PazPC, and then treated with free drug, drug uptake did not increase (Supplemental Fig. 1A and B). Furthermore, no, or only slightly enhanced cellular uptake was achieved by simply mixing the drugs and PazPC in the cell culture medium. Probably, the low enhancement of drug uptake induced by the simple mixing of drug and PazPC resulted from the spontaneous formation of ion–pair complexes and micelles between PazPC and drug in the cell culture medium due to its neutral pH (pH 7.0–7.5). This phenomenon of a low enhancement induced by a mixture of drug and PazPC was further confirmed by the MTT assay. The IC_{50} of a simple mixture of free drug and PazPC on both resistant P388/ADR and sensitive P388 cells is on the same order of magnitude as that of free DOX or IDA (Supplemental Fig. 2). These observations suggest that in order to achieve enhanced DOX or IDA cell uptake, the drug must be entrapped in the PazPC micelles. Some oxidized phospholipids (OxPLs), such as PazPC, account for almost two-thirds of oxidized phospholipids in oxidized LDL (OxLDL) (Chen et al., 2007). Scavenger receptors on the surface of macrophages recognize OxPLs in OxLDL and mediate the uptake of OxLDL (Fruhwirth et al., 2007; Bochkov et al., 2010). Considering that murine leukemia P388 is a neoplastic cell of macrophage lineage (Bauer et al., 1986; Lechleitner et al., 1994), it is highly possible that the PazPC-based micellar system may exert its advantage on drug uptake and cytotoxicity on these cell lines through this scavenger receptor. Interestingly, the micelle drug formulations did not enhance the uptake of drugs in normal bone marrow cells (Supplemental Fig. 3), suggesting specificity of this system for leukemia treatment. A recent report also demonstrated negatively charged liposome mediated selective accumulation of cytotoxic agents within leukemic cells residing in the bone marrow through the scavenger receptor (Lim et al., 2010). Importantly, increased expression of scavenger receptors have been reported in tumor cells of leukemia patients (Belov et al., 2001; Xu et al., 2006; Banker et al., 2004) and in leukemia cells (Scherf et al., 2000).

The resistant P388/ADR cell is more susceptive to drug-loaded PazPC micelles than the sensitive P388 cell with regard to both uptake and cytotoxicity. These differences between P388 and P388/ADR are probably due to a different pattern of uptake, intracellular retention and distribution, as well as subsequent biological effects among free drug, PazPC micelle, and the ion–pair complexes of PazPC and drug. So far, it is not clear how the PazPC micelles enhance drug uptake and cytotoxicity in leukemia cells. However, oxidized phospholipids are a type of endogenous substances that exhibit a variety of biological activities (Bochkov et al., 2010). PazPC can interact with mitochondria and lead to cytochrome c and apoptosis-inducing factor releasing from mitochondria to the cytoplasm and nucleus, respectively (Chen et al., 2007, 2009). Mitochondria damage can not only initiate apoptosis cascade, but also suppress the production of ATP. The former effect can probably kill the tumor cell synergistically with anti-tumor drug, and the latter can subsequently affect the function of ATP-dependent MDR-related proteins, such as P-gp, resulting in increasing intracellular drug. Furthermore, some OxPLs, e.g., OxPAPC (oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine), can increase intracellular levels of reactive oxygen species (ROS) (Bochkov et al., 2010), and cancer cells would be more vulnerable to further oxidative stress induced by exogenous ROS-generating agents (Trachootham et al., 2009). The increased ROS level in P388 and P388/ADR cells by PazPC was observed in our lab (data not shown). Probably, increased ROS induced by PazPC in leukemia cells could be another mechanism to kill cancer cells synergistically with the drug. The detail mechanisms of PazPC micelles to induce enhanced uptake and cytotoxicity of DOX and IDA in leukemia cells are under investigation in our lab.

5. Conclusion

We designed and developed a novel micellar formulation, constructed by a single oxidized phospholipid PazPC for delivery of anthracycline anticancer drugs, DOX and IDA. The ion–pair complexes between PazPC and DOX were effectively formed and subsequently the drug-loaded PazPC micelles were prepared at a 5:1 molar ratio of lipid to drug, with high encapsulation efficiency (62% for DOX, 87% for IDA), small particle size (8–10 nm) and good stability at physiological conditions. The drug-loaded PazPC micelles were sensitive to pH change with accelerated release of DOX or IDA in an acidic environment. Importantly, they enhanced drug uptake and exhibited a higher cytotoxicity effect on both sensitive and resistant leukemia cells in comparison to free drug. Therefore, the oxidized phospholipid-based micelle system could be a promising carrier for anthracycline anticancer drugs and improve the therapeutic outcomes of leukemia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.10.029.

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