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Nanohybrid systems of non-ionic surfactant inserting liposomes loading paclitaxel for reversal of multidrug resistance

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ABSTRACT

Three new nanohybrid systems of non-ionic surfactant inserting liposomes loading paclitaxel (PTX) (NLPs) were prepared to overcome multidrug resistance (MDR) in PTX-resistance human lung cancer cell line. Three non-ionic surfactants, Solutol® HS 15 (HS-15), pluronic F68 (PF-68) and cremophor EL (CrEL) were inserted into liposomes by film hydration method to form NLPs with an average size of around 110, 180 and 110 nm, respectively. There was an obvious increase of rhodamin 123 (Rh123) accumulation in A549/T cells after treated with nanohybrid systems loading Rh123 (NLRs) when compared with free Rh123 or liposomes loading Rh123 without surfactants (LRs), which indicated the significant inhibition effects of NLRs on drug efflux. The P-gp detection and ATP determination demonstrated that BNLs could not only interfere P-gp expression on the membrane of drug resistant cells, but also decrease ATP level in the cells. The cytotoxicity of NLPs against A549/T cells was higher than PTX loaded liposomes without surfactants (LPs), and the best result was achieved after treated with NLPs2. The apoptotic assay and the cell cycle analysis showed that NLPs could induce more apoptotic cells in drug resistant cells when compared with LPs. These results suggested that NLPs could overcome MDR by combination of drug delivery, P-gp inhibition and ATP depletion, and showed potential for treatment of MDR.

1. Introduction

Multidrug resistance (MDR) is one of the major causes of chemotherapeutic failures. MDR can be intrinsic or acquired after chemotherapy with the phenotype of cross-resistance against several unrelated drugs with different molecular structure and target specificity (Ozben, 2006; Szakács et al., 2006). One of the most common mechanisms of MDR is drug efflux mediated by efflux transporters such as P-glycoprotein (P-gp), which is one of the most typical and the most widely investigated members of ATP-binding cassette (ABC) transporters. It has been reported that P-gp could use the energy of ATP-hydrolysis to pump substrates such as doxorubicin and paclitaxel (PTX) out of tumor cells, which resulted in the reduction of accumulation of drugs in tumor cells (Gottesman et al., 2002). To address this problem, some reversal agents that inhibited MDR efflux proteins have been investigated (Tan et al., 2000). Unfortunately, these agents showed low efficiency with high toxicity, and the results of the clinical trails were disappointing (Lee, 2010; Leonard et al., 2002). Compared with blockers of drug resistant proteins, nanoparticles-based drug delivery systems, such as lipid nanoparticles and liposomes, have attracted great attention because they could enhance cellular uptake, increase intracellular drug concentration, avoid the disadvantages of inherent toxicity caused by the inhibitors, and bypass trans-membrane protein mediated efflux (Liang et al., 2010; Jabr-Milane et al., 2008). In addition, the nanoparticles loading drug could act as intracellular anti-cancer drug reservoirs after entering the tumor cells, which could protect drug from degradation and increase drug efficacy. However, most of the free intracellular drug was still extruded by efflux protein because nanoparticles themselves could not inhibit the function of membrane efflux protein (Wong et al., 2006; Chavanpatil et al., 2006).

Non-ionic surfactants have been widely used in pharmaceutical field as solubilizers and stabilizers of insoluble drugs due to their surface activity and low toxicity (Katakam et al., 1995; Kerwin, 2008), and a few of surfactants showed the ability to reverse active transport mediated by multidrug resistance proteins, such as cremophor EL (CrEL) and Tween 80, which could inhibit P-gp activity in Caco-2 cell monolayers (Hugger et al., 2002). The non-ionic surfactant Solutol® HS 15 (HS-15) showed to reverse the MDR of human epidermoid carcinoma cells in vitro, but did not potentiate drug toxicity in drug-sensitive cells (Coon et al., 1991). Pluronic exhibited the ability to sensitize MDR tumor by inhibiting P-gp drug efflux system by ATP depletion (Batrakova et al., 2010).

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We are interested in developing a new drug delivery system to reverse MDR. In this work, a new nanohybrid system by non-ionic surfactant inserting liposomes loading PTX (NLPs) was designed and prepared. It was postulated that this nanohybrid system could reverse MDR and increase drug efficacy efficiently with the advantages of effective intracellular drug delivery and simultaneous P-gp inhibition. In present work, three non-ionic surfactants, HS-15, pluronic F68 (PF-68) and CrEL, were selected to insert into liposomes to construct NLPs, and the effects of different non-ionic surfactants on the physicochemical characteristics of NLPs were investigated. The P-gp function, P-gp expression and the intracellular ATP level were determined in a PTX resistant human lung carcinoma cell line (A549/T) to investigate the role of surfactants in this nanohybrid system in the reversal of MDR. The cytotoxicity of NLPs against PTX sensitive and resistant A549 cells was examined by MTT assay, and the apoptosis assay and cell cycle analysis were also performed to determine the effects of NLPs on the reversal of MDR.

2. Materials and methods

2.1. Materials

PTX (>99%) was purchased from Sunve Pharmaceutical Co., Ltd. (Shanghai, China). HS-15, PF-68 and CrEL were obtained from BASF (Ludwigshafen, Germany). Soybean lecithin was purchased from Lipoid® GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Trypsin–EDTA and phosphate buffered solution (PBS) were obtained from Gibco-BRL (Burlington, ON, Canada). The RPMI 1640 medium, antibiotics and Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit were purchased from Invitrogen (Oregon, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Rh123, DNA-free RNase A and propidium iodide (PI) were purchased from Sigma (St. Louis, USA). ATP assay kit was purchased from Beyotime® Institute of Biotechnology (Shanghai, China). Phycoerythrin (PE)-anti-human MDR1 (CD243, P-gp, ABCB1) and PE-Mouse IgG2a (κ Isotype Control) were obtained from eBioscience (CA, USA). All other chemicals and solvents if not mentioned were of analytical grade and used as received without additional purification.

2.2. Cell culture

The human lung adenocarcinoma epithelial cell line (A549) and its PTX-resistant derivative cell line (A549/T) were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate and maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.3. Preparation of nanohybrid systems of non-ionic surfactant inserting liposomes loading PTX

Nanohybrid systems of non-ionic surfactant inserting liposomes loading PTX (NLPs) were prepared by thin-film hydration method. Briefly, PTX, soybean lecithin, cholesterol and surfactant at mass ratio of 1:30:10:20, were dissolved in 5 mL dichloromethane. The organic solvent flask was attached to a rotary evaporator (Heidolph Laborata 4000, Germany) to form lipid film, then the film was hydrated with 10 mL PBS in 50 °C water bath for 20 min. The resultant suspension was sonicated for 4 min (200 W, 2 min; 400 W, 2 min) with a probe (JYD-650, Shanghai, China) to form NLPs, and filtrated through 0.22 μm microporous membrane. As control, PTX loaded liposomes without surfactants (LPs), blank nanohybrid systems without loading PTX (BNLs) and blank liposomes without surfactants (BLs) were prepared by the same procedure as described above. In Rh123 efflux experiment, nanohybrid systems of non-ionic surfactant inserting liposomes containing Rh123 (NLRs) and Rh123 loaded liposomes without surfactants (LRs) were prepared with the same procedure by usage of Rh123 as replacement of PTX. The NLPs were finally freeze-dried in the presence of sucrose and stored at 4 °C for further experiments.

2.4. Physicochemical characteristics of NLPs

The mean particle size and zeta potential of NLPs were determined by dynamic light scattering method using Malvern zetasizer (Nano-ZS90, Malvern Instruments Ltd., UK). The amount of PTX incorporated in NLPs was determined by HPLC with following conditions: Agilent Eclipse XCB C_{18} column (150 mm × 4.6 mm i.d., pore size 5 μm); the mobile phase, CH_3CN:H_2O (70:30, v/v); flow rate, 1.0 mL/min; the measured wavelength: 227 nm. The encapsulation efficiency (EE) was measured immediately after preparation using centrifugation and ultracentrifugation method (Yang et al., 2007) and calculated as below:

$$EE\,(\%) = \frac{P_{rec}}{P_{ini}} \times 100\% \quad (1)$$

P_{rec}: PTX recovered from NLPs; P_{ini}: PTX initially used.

In order to know the in vitro release behavior of PTX, the dialysis method was applied to monitor the release of PTX from NLPs at the presence of Tween 80 (0.1% in PBS, pH 7.4) as described previously (Yang et al., 2007). The sample volume in dialysis bag (cutoff Mw = 7 kDa) was 0.5 mL at 1 mg/mL, and the sink solution was 200 mL of release medium. The dialysis was carried out at 37 °C, and the concentration of drug was analyzed by HPLC as described above at various time points during the dialysis process.

2.5. Rh123 efflux assay for P-gp function

The accumulation of Rh123 in A549 and A549/T cells was first detected by fluorescence microscopy. A549 and A549/T cell monolayers were cultured on 10 mm^2 glass coverslips for 24 h. After incubation with free Rh123 solution, LRs or different NLRs (5 μg/mL Rh123) for 3 h at 37 °C, cells were washed three times with PBS (pH 7.4). Then, cells were incubated with fresh growth medium for 1 h to allow efflux of the intracellular Rh123. Subsequently, cells were observed under a fluorescence microscope (Olympus RX81, Japan). Rh123 was excited using the 488-nm line, and the fluorescence microscope parameters were set up so that the cells in the well without Rh123 did not produce fluorescent signal.

The accumulation of Rh123 in A549/T cells was then measured quantitatively. A549/T cells were seeded in a 24-well plate with 0.5 mL growth medium and allowed to attach for 24 h. Then, cells were incubated with free Rh123 solution, LRs or different NLRs (5 μg/mL Rh123) for 3 h at 37 °C, followed by washing three times with PBS (pH 7.4). Cells were incubated with fresh growth medium for 1 h to allow efflux of the intracellular Rh123. Finally, cells were detached, subjected to flow cytometry and analyzed with CellQuest software.

2.6. Effect of BNLs on P-gp expression

A549/T cells were seeded in 24-well plates at a density of 5×10^4/well with 0.5 mL growth medium and allowed to attach for 24 h. The medium was replaced with fresh growth medium containing BLs or three BNLs without PTX (150 μg/mL soybean lecithin). After 24 h incubation, cells were trypsinized, collected and resuspended in PBS (pH 7.4). PE-conjugated mouse anti-human monoclonal antibody against P-gp was used to label cells according to manufacturer's instruction, and the nonspecific labeling was corrected by its isotype control. The cell resuspension was finally

subjected to a FACSCalibur system (Beckton Dickinson, USA) and analyzed with CellQuest software through fluorescence channel 2 (FL2).

2.7. Effect of BNLs on intracellular ATP levels

The ATP level was measured by the luciferin–luciferase method following the protocol of ATP assay kit (Beyotime, China). Cells were seeded in 24-well plates at a density of 1×10^5/well with 0.5 mL growth medium and incubated overnight. Following treatment with BLs or three BNLs without loading PTX (150 μg/mL soybean lecithin) for 24 h, cells were washed with ice-cold PBS and lysed with ATP lysis buffer. ATP in cell lysate was measured using a POLARstar OPTIMA microplate reader (BMG Labtech, Germany) by calibration with the ATP standards.

2.8. In vitro cytotoxicity experiment

A549 and A549/T cells were cultured on 96-well plates at a density of 8000 cells/well. The cells were incubated for 24 h to allow for attachment to the culture vessel before they were washed with prewarmed sterile PBS (pH 7.4), followed by exposition to free PTX, BNLs, LPs or three NLPs diluted with culture medium to various concentration for 48 h at 37 °C. Then cell viability was evaluated by MTT assay. The amount of MTT formazan product was analyzed spectrophotometrically at 570 nm using an automated plate reader (Tecan Spectrafluor Plus, Austria). All drug concentrations were tested in six replicates.

2.9. Apoptosis assay and cell cycle analysis

For the analysis of apoptosis, about 1×10^6 A549/T cells were treated with free PTX solution or NLPs for 24 h with 5 μg/mL. After incubation, cells were stained using the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit according to the manufacturer's protocol. The stained cells were analyzed using the FACSCalibur system (Becton Dickinson, USA) with CellQuest software.

Cell cycle was assessed by flow cytometry. Briefly, A549/T cells seeded on the plate were treated with free PTX solution or NLPs for 24 h with 5 μg/mL. After incubation, adherent and non-adherent cells were recovered. Cells (1×10^6) were collected by centrifugation, washed twice with PBS, and then fixed with 70% pre-cooled ethanol and stored at 4 °C for 22 h. Cells were centrifuged again, washed with cold-PBS twice and incubated with RNase A (10 mg/mL) for 20 min at 37 °C, and stained with PI (2 mg/mL) for 30 min in the dark. The DNA content was measured by FACSCalibur system (Becton Dickinson), and the percentage of cells in each phase of the cell cycle was evaluated using the ModFit software (Verity Software House, Topsham, ME).

2.10. Statistical analysis

Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for $p < 0.05$ and $p < 0.01$ indicative of a very significant difference.

3. Results and discussion

3.1. Preparation and characterization of NLPs

The NLPs were prepared by a conventional thin-film hydration method. The schematic illustration of the non-ionic surfactant-lipid nanohybrid systems was shown in Fig. 1. The nanohybrid systems with different composition or rate were prepared in advance, and NLPs with PTX, soybean lecithin, cholesterol and surfactant with a mass ratio of 1:30:10:20 showed the optimal particle size and high encapsulation efficiency (data not shown). The three surfactants, HS-15, PF-68 and CrEL were selected to prepare NLPs, which were named NLPs1, NLPs2 and NLPs3, respectively. The physicochemical properties of NLPs including particle size, polydispersity index (PDI), surface potential and encapsulation efficiency were shown in Table 1. NLPs showed larger particle size than BNLs, which indicated that PTX loaded into nanohybrid systems could slightly expand the particles. NLPs1 and NLPs3 showed smaller particle size than LPs, which could be due to the enhanced hydrophilicity of NLPs surface by the polyoxyethylene chains (Zhang et al., 2008), but the insertion of PF-68 could increase particle size. These results demonstrated that the insertion of tri-block copolymer into lipid vesicles could slightly reduce the structural integrity of bi-layer vesicles, while di-block copolymer could enhance the structural integrity of NLPs. NLPs showed higher PDI than LPs, which demonstrated that the insertion of surfactants could widen the size distribution

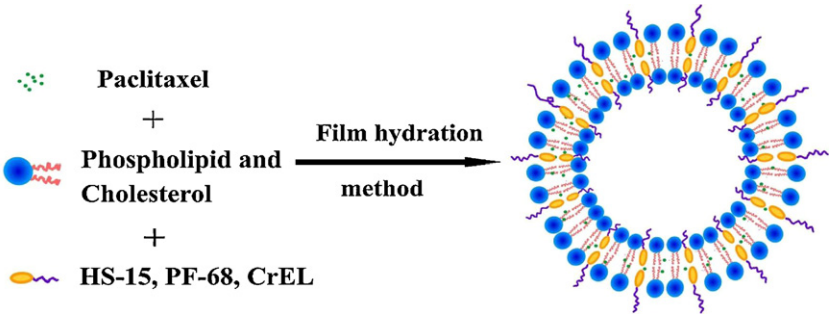

Fig. 1. Schematic illustration of assembly of NLPs.

Table 1
Physicochemical properties of BNLs and NLPs ($n = 3$).

	Mean particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Encapsulation efficiency (%)
LPs	139.8 ± 1.4	0.274 ± 0.007	−3.67 ± 0.11	98.20 ± 0.56
BNLs1	89.9 ± 2.3	0.228 ± 0.004	−6.27 ± 0.14	
NLPs1	108.8 ± 4.0	0.350 ± 0.002	−4.61 ± 0.08	98.38 ± 0.17
BNLs2	160.5 ± 3.7	0.273 ± 0.012	−2.74 ± 0.15	
NLPs2	181.0 ± 2.0	0.305 ± 0.007	−2.34 ± 0.24	97.60 ± 0.33
BNLs3	92.5 ± 1.6	0.216 ± 0.009	−5.46 ± 0.21	
NLPs3	111.2 ± 2.7	0.332 ± 0.014	−4.52 ± 0.16	98.69 ± 0.53

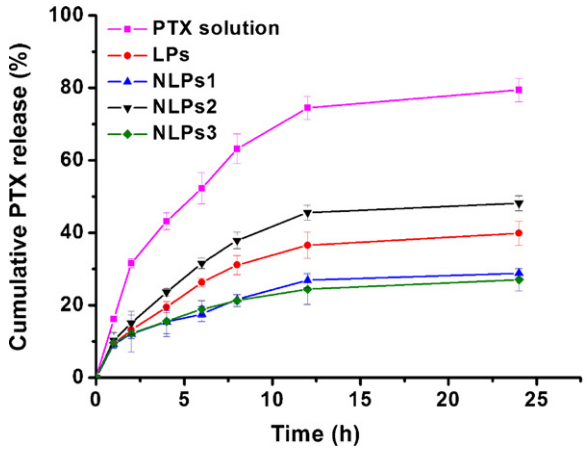

Fig. 2. The in vitro release profiles of LPs and NLPs in PBS (pH 7.4) containing 0.1% (v/v) Tween 80 at room temperature. PTX in a mixture of cremophor EL and ethanol (50:50, v/v) solution was used as a free drug reference for diffusion across the dialysis membrane. Each data represents the mean ± SD ($n = 3$).

of lipid vesicles. The size distribution of NLPs comprised only one peak, which indicated that the surfactants were inserted into the lipid vesicles instead of forming micelles outside lipid vesicles. NLPs showed similar zeta potential and drug encapsulation efficiency to LPs, which indicated that the insertion of surfactants into lipid vesicles did not significantly affect surface charge and drug encapsulation efficiency of lipid vesicles.

3.2. Release behavior of NLPs

The nanohybrid systems of liposomal cerasomes (Cao et al., 2010) or diacetylene/phospholipid polymerized vesicles (Guo et al., 2009) have been designed for PTX delivery, and the nanohybrid systems are capable of regulating the release behavior of anticancer drugs. So the release behaviors of LPs and NLPs were compared to find the effects of surfactants on the release behaviors of lipid vesicles. Due to the poor water solubility of PTX, it is one of the difficulties to maintain sink condition in carrying out the in vitro release experiments. It was reported that the solubility of PTX could achieve to 6.32 μg/mL in PBS containing 0.1% (v/v) Tween 80 (Yang et al., 2007), and Tween 80 solution was used as release medium to investigate the release behaviors of some formulations of PTX (Koziara et al., 2004; Zhang et al., 2004). In present work, 0.1% (v/v) Tween 80 was added to the release medium (PBS, pH 7.4) to maintain sink condition during the release experiment. Because of the delayed diffusion through the dialysis membrane, PTX in a mixture of CrEL and ethanol (50:50, v/v) was used as control. The in vitro release profiles of LPs and NLPs were shown in Fig. 2. PTX in the solution released rapidly with the cumulative release about 80% within 24 h. LPs and NLPs showed an initial burst release phase followed by a sustained release profile. Because PTX showed good affinity for lipid materials due to its high lipophilicity, LPs released only 39.9% of PTX within 24 h. NLPs showed obvious change in drug release profiles compared with LPs, which suggested that the modification of lipid vesicles with surfactants could affect the structure integrity and in vitro stability of lipid vesicles. NLPs1 and NLPs3 demonstrated much slower release rate than LPs with cumulative PTX release of 29.1% and 28.9%, respectively, which indicated that insertion of HS-15 or CrEL into the structure of lipid bilayer could stabilize lipid vesicles. However, NLPs2 showed faster release rate than LPs, which indicated that the insertion of tri-block copolymer into lipid vesicles could slightly reduce the structural integrity of bi-layer vesicles, and resulted in increased release rate of incorporated drug. This result suggested that PF-68 molecules were not tightly inserted into lipid vesicles, which was consistent with the physicochemical properties of NLPs2.

3.3. Rh123 efflux assay

A number of therapeutic drugs including PTX have been proved to be P-gp substrates. P-gp function could be determined by a cell-permeant fluorescent dye Rh123, which is a substrate of P-gp. It was reported that P-gp function was significantly correlated with Rh123 efflux, and the inhibition of P-gp could result in dye retention (Saengkhae et al., 2003). Due to the appropriate hydrophilic and hydrophobic property, Rh123 could be almost completely entrapped into the nanohybrid systems in this experiment.

The Rh123 efflux abilities of A549 and A549/T cells were first observed by fluorescence microscopy. As shown in Fig. 3A, Rh123 fluorescence distributed fully inside A549 cells, but was scarcely found in A549/T cells treated with free Rh123, which indicated a strong efflux of Rh123 in A549/T cells. LRs group showed no obvious improvement in accumulation of Rh123 inside drug sensitive cells compared with free Rh123 treated group, however, the dye retention was markedly enhanced when entrapped in LRs in drug resistant cells. The inhibited Rh123 efflux in A549/T cells treated with LRs could result from the enhanced cellular delivery by endocytosis and bypassing membrane-protein mediated efflux. Both drug sensitive and resistant cells demonstrated higher Rh123 accumulation after treated with NLRs compared with LRs, which indicated that the insertion of non-ionic surfactants into lipid vesicles could decrease P-gp efflux rate.

The efflux inhibition effects of NLRs in drug resistant cells were also determined quantitatively by FACS assay (Fig. 3B and C). The mean fluorescent intensity of cells treated with free Rh123 was similar to that of control cells, but the cells treated with LRs or NLRs all demonstrated high mean fluorescent intensity. Compared with LRs, NLRs could significantly increase mean fluorescent intensity in cells, which suggested the strong inhibition effects of surfactants on P-gp mediated efflux. The highest mean fluorescent intensity was obtained in NLRs1 treated group, which was consistent with the observed results that NLRs1 treated A549/T cells showed strong fluorescence, which was even higher than that in drug sensitive cells. NLRs2 with surfactant PF-68 showed the lowest dye retention in cells among three NLRs, which could be due to the poor stability of nanohybrid systems. NLRs1 and NLRs3 with better stability and slower drug release rate could facilitate cellular uptake and reduce drug efflux.

3.4. Determination of P-gp expression

It was reported that some anti-tumor drug could increase the intracellular accumulation of Rh123 without affecting the expression of P-gp (Zhang et al., 2010). The enhanced Rh123 uptake in hepatocytes did not result from reduced expression of P-gp, but from dysfunction of the efflux transporter (Akazawa et al., 2002). Therefore, it could not be determined that the surfactants in the lipid formulation could affect P-gp expression, and thus cause the inhibited Rh123 efflux in drug resistant cells. To know whether these three surfactants had P-gp inhibition effect and the decreased drug efflux rate was result partly from reduced expression of P-gp, the expression of P-gp on membrane of A549/T cells was determined by flow cytometry using fluorescently labeled P-gp antibody. To minimize the effect of entrapped fluorescent dyes or drugs, cells were treated with BLs or BNLs without loading dye or drug. From the histograms in Fig. 4, it could be found that BLs treated cells showed no change on P-gp expression, but P-gp expression slightly decreased on cells treated with BNLs. As surfactant was the only difference between BLs and BNLs, it was no doubt that the surfactants had P-gp inhibition effects.

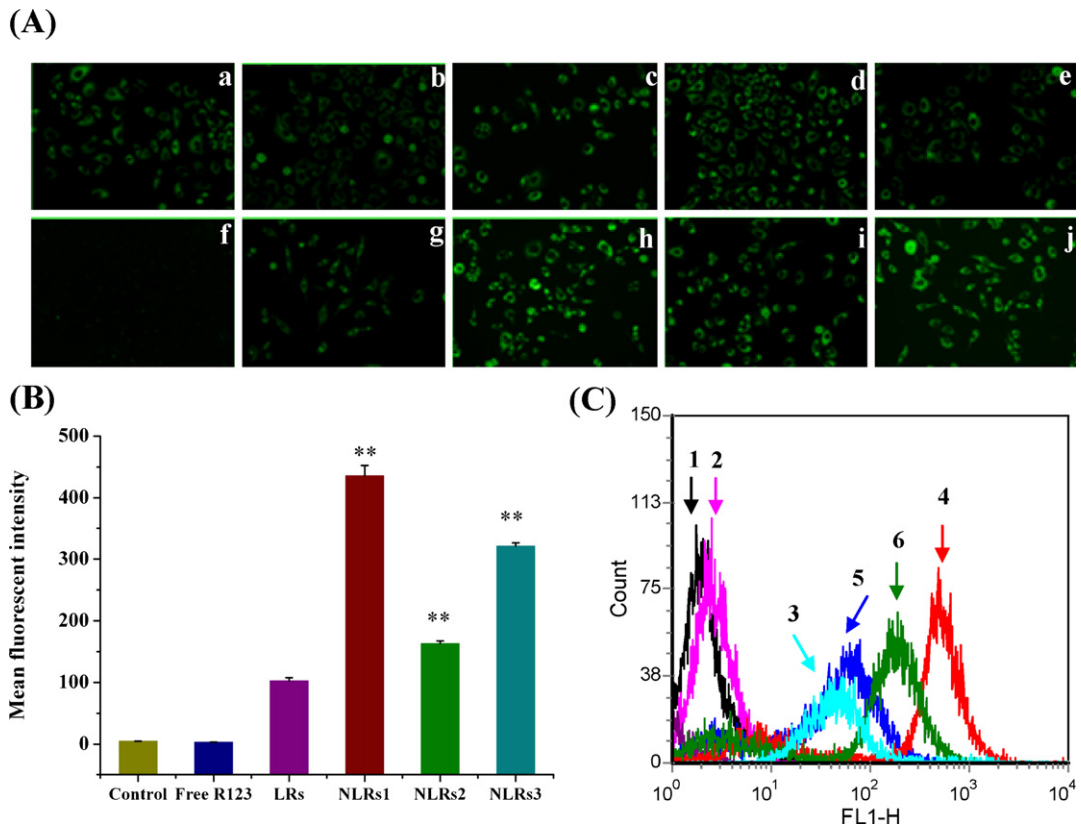

Fig. 3. Rh123 efflux experiment. (A) Images of A549 (a–e) and A549/T (f–j) cells treated with free Rh123, LRs or NLRs for 3 h, followed by 1 h efflux. a and f: Treated with free Rh123; b and g: treated with LRs; c and h: treated with NLRs1; d and i: treated with NLRs2; e and j: treated with NLRs3. (B) Quantitative analysis of mean fluorescent intensity of A549/T cells after treated with Rh123 by FACSCalibur flow cytometry. $^{**}p < 0.01$ compared with RLs group. (C) The FCM pictures of control cells (1), cells treated with free R123 (2), LRs (3), NLRs1 (4), NLRs2 (5) or NLRs3 (6) of collected A549/T cells.

It is worth mentioning that P-gp was not over-expressed on A549/T cells in this work, which was accordant with previous other report that PTX-resistant A549 cell lines expressed low amounts of P-gp (Yang et al., 1998). Therefore, the effects of surfactants on P-gp expression could not be obviously found from the histograms. However, compared with the isotype control and the results from drug sensitive cells, P-gp was expressed on A549/T cells, and higher than that on A549 cells. Although some reports considered that tubulin

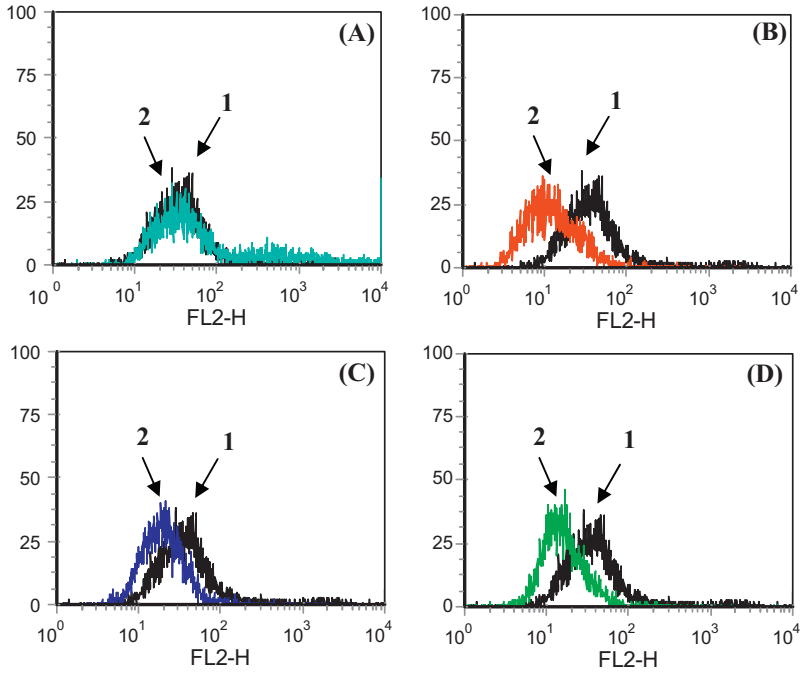

Fig. 4. P-gp expression on A549/T cell membrane before (1) and after (2) treated with BLs (A), BNLs1 (B), BNLs2 (C) or BNLs3 (D).

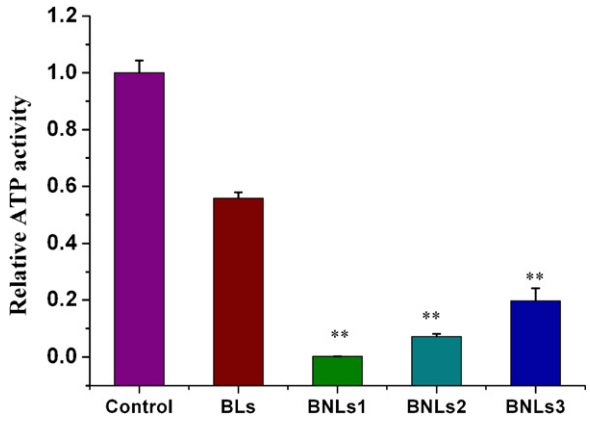

Fig. 5. ATP depletion in A549/T cells. Cells were treated with BLs or BNLs without PTX for 48 h. $^{**}p < 0.01$ compared with BLs group.

mutation and increased microtubule instability could be the reasons for resistance of PTX in A549/T cells (Goncalves. et al., 2001; Martello et al., 2000, 2003), the significant difference in the expression of P-gp between PTX-sensitive and PTX-resistant A549 cells was still found (Sun et al., 2010). We thought that the properties of A549/T cells were associated with the drug concentration required for the maintenance of drug resistance. Cells cultured with different drug concentration might demonstrate different drug resistant property. Therefore, it was suggested that P-gp could be an important factor for the resistance of A549/T cells based on our study.

3.5. Determination of ATP level

The over-expression of ATP-dependent membrane transporter proteins in cancer cells has been considered as one of the main causes of MDR (Liang et al., 2010; Borowski et al., 2005). Because drug efflux is an energy-dependent process, the intracellular ATP levels of cells after treated with BLs or BNLs without loading drug were investigated. It was found that cells exposure of BLs could induce a decrease in ATP level in drug resistant cells (Fig. 5). This phenomenon was also reported in blank lipid-based nanoparticles, which could deplete ATP in drug resistant human ovarian carcinoma NCI/ADR cells (Dong et al., 2009). This could be due to the cellular uptake of lipid vesicles was an energy-consuming process. Cells treated with three BNLs all showed lower ATP activity than cells treated with BLs, which indicated that the insertion of non-ionic surfactant into liposomes could significantly decrease ATP level in drug resistant tumor cells. The cells treated with BNLs1 demonstrated the weakest ATP activity, which indicated the strongest depletion effect of HS-15 on ATP. The results of P-gp detection and ATP determination demonstrated that liposomes containing non-ionic surfactants could not only interfere P-gp expression on the membrane of drug resistant cells, but also decrease ATP level in the cells. The results suggested that employing the nanohybrid systems could reverse drug resistance more effectively in MDR cells.

3.6. In vitro cytotoxicity of NLPs in sensitive and resistant cells

The dose-response of PTX in LPs or NLPs against sensitive and resistant cells was shown in Fig. 6. After incubation with 20 μg/mL free PTX for 48 h, about 55% of A549 cells were inhibited, while the cell viability of A549/T cells was over 75% after treated with 20 μg/mL free PTX, which indicated the resistance of PTX against A549/T cells. In drug sensitive cells, the encapsulation of PTX by LPs could not enhance the cytotoxicity of PTX. LPs could slightly enhanced cytotoxicity against drug resistant cells, which demonstrated that LPs could reverse the PTX resistance of A549/T cells to some extent. The enhanced endocytosis in drug resistant cells mediated by nanohybrid systems could be the main reason for enhanced cytotoxicity. BNLs did not show obvious toxicity (data not shown).

The toxicities of nanohybrid systems after insertion of surfactants in A549 and A549/T cells were very different. NLPs1 and NLPs3 showed enhanced toxicity in A549 cells than LPs, but the toxicity of NLPs2 was lower than LPs, which could result from the instability of NLPs2 structure and the burst drug release behavior of NLPs2, which led to lower intracellular drug concentration. In drug resistant cells, the surfactants inserting liposomes all could increase cytotoxicity. We speculated that the surfactants not tightly bound to lipid vesicle surface could release from the bilayer vesicle with time inside the cells. The free surfactants could inhibit the function of P-gp, and in the mean time, form micelles with PTX, both of which could reduce intracellular drug efflux and enhance drug toxicity. These results demonstrated that NLPs could combine the advantages of drug delivery and P-gp inhibition to reverse MDR. In drug resistant cells, NLPs2 showed the highest toxicity among the three formulations. The unique effects of pluronic copolymer in MDR cells could be the reasons for the best reversion effect of NLPs2. In addition to the P-gp inhibition and ATP depletion, it was reported that pluronic block copolymers could induce the increase of reactive oxygen species levels in the cytoplasm (Batrakova and Kabanov, 2008), inhibit the glutathione/glutathione S-transferase detoxification system (Yamagata et al., 2007), and enhance apoptotic signaling in MDR cells (Minko et al., 2005; Batrakova et al., 2010). The enhanced cytotoxicity of NLPs2 in drug resistant cells could result from the synergistic effects of PTX and PF-68.

3.7. Quantitative apoptotic assay and cell cycle analysis

PTX binds to the beta-subunit of the alphabeta-tubulin dimer in the microtubule, induces the polymerization of tubulin and causes the growth arrest of rapidly dividing cells, thereby leads to

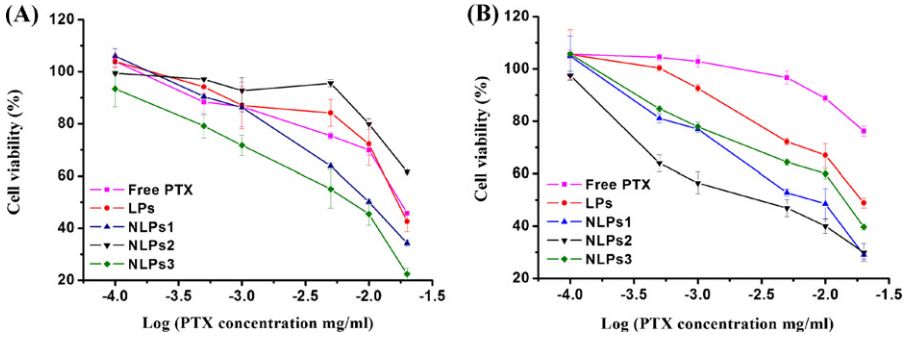

Fig. 6. In vitro cytotoxicity of different paclitaxel-loaded NLPs against A549 (A) and A549/T (B) cells.

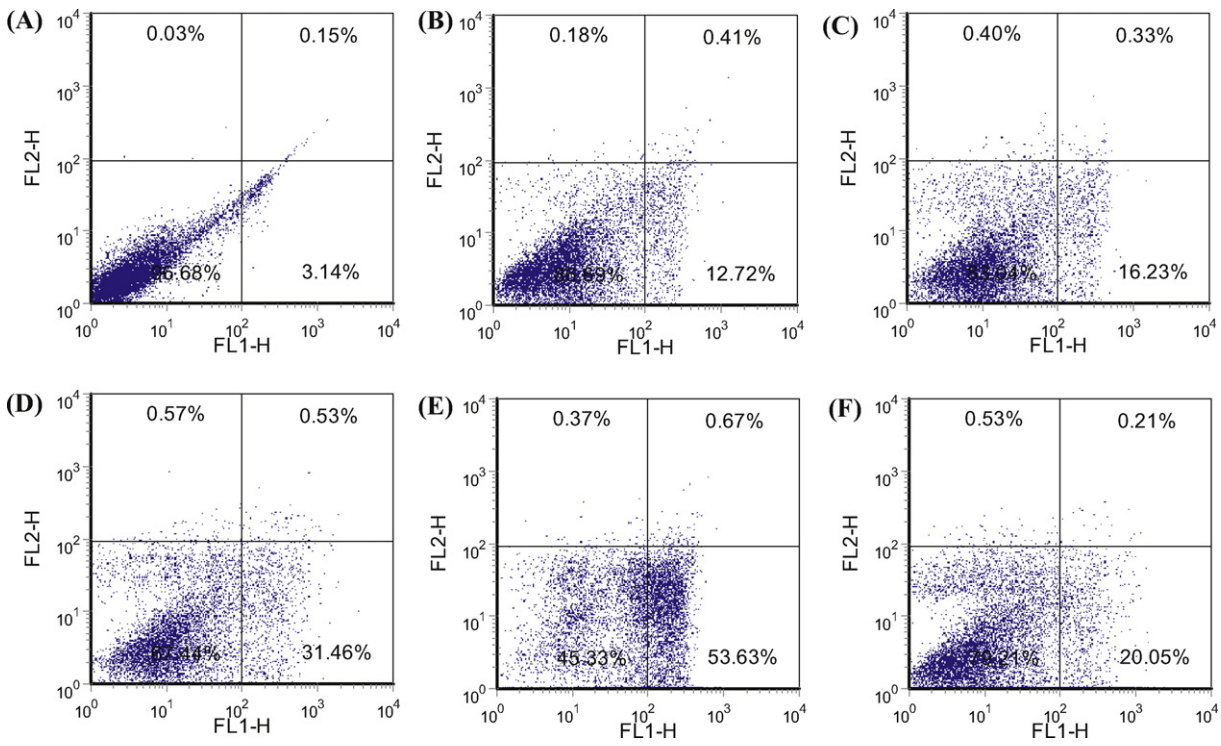

Fig. 7. Apoptosis analysis of A549/T cells after treated with NLPs. (A) Control; (B) treated with free PTX; (C) treated with LPs; (D) treated with NLPs1; (E) treated with NLPs2; (F) treated with NLPs3.

apoptosis (Ganesh et al., 2007). It was reported that 10 μmol/L of PTX could induce apoptotic cell death and arrest cells in the G2/M phase of the cell cycle in different lung cancer cell lines such as A549, H226, H596 and 84T (Weigel et al., 2000). To confirm that NLPs could enhance the toxicity of PTX and reverse MDR in A549/T cells, the quantitative apoptotic assay was performed. The cells treated with 5 μg/mL PTX for 48 h were co-stained with Alexa Fluor® 488 annexin V and PI for cytoflow analysis. Due to drug resistance, free PTX induced only 12.72% of apoptotic cells (Fig. 7). Encapsulation of PTX into LPs could increase apoptotic cells to 16.23%, which indicated the bypassing effect of drug efflux by LPs. PTX entrapped in this nanohybrid systems could induce more apoptosis, and the severe apoptosis was found in cells treated with NLPs2 with over a half of cells apoptosis.

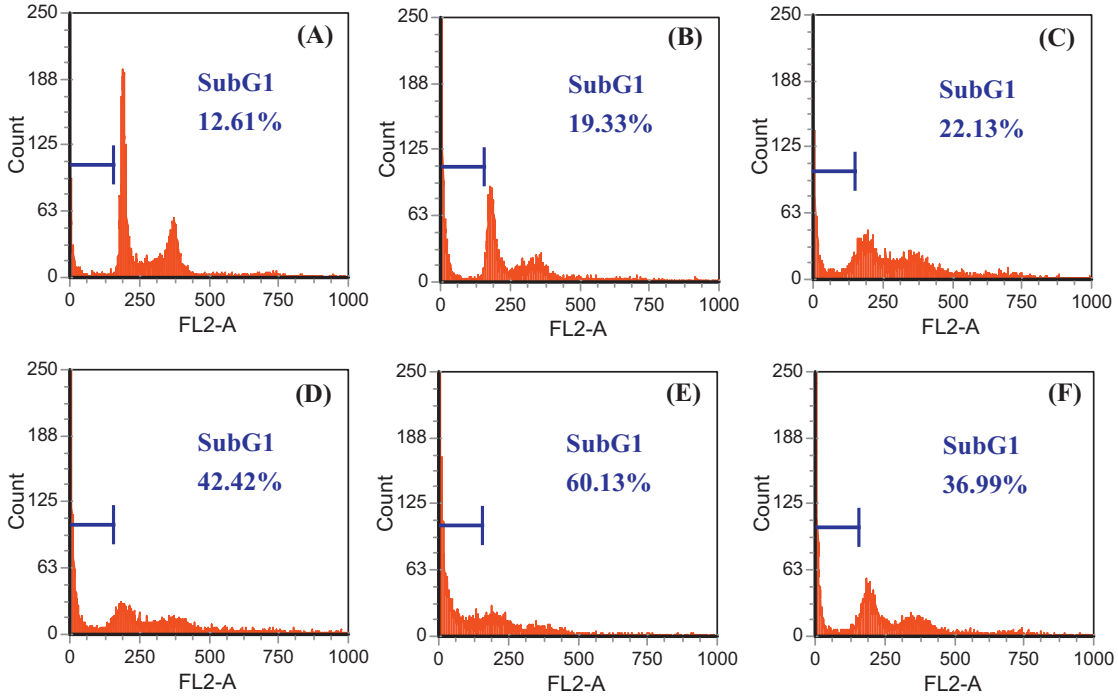

Fig. 8. Cell cycle analysis of A549/T cells after treated with NLPs. (A) Control; (B) treated with free PTX; (C) treated with LPs; (D) treated with NLPs1; (E) treated with NLPs2; (F) treated with NLPs3.

The cycle of cells was also analyzed to investigate the toxicity of PTX entrapped in NLPs and the reverse effect of NLPs in A549/T cells. The results from cell cycle analysis showed the presence of a subG1 apoptotic population after treated with formulations of PTX (Fig. 8). It demonstrated that PTX entrapped into this nanohybrid system could improve toxicity of PTX, and NLPs induced significantly more subG1 population than LPs, which indicated that the insertion of surfactants in lipid vesicles could significantly increase PTX toxicity and reverse PTX resistance. These results were consistent with above cytotoxicity study and apoptotic assay. The improved toxicity of NLPs in drug resistant cells compared with LPs could be due to the effects of non-ionic surfactants on P-gp inhibition and ATP depletion, thus to reduce drug efflux and increase intracellular drug accumulation. Among three NLPs formulations, NLPs2 demonstrated the most cytotoxic, while NLPs3 was the least cytotoxic. The marked toxicity of NLPs2 against A549/T cells could result from the enhanced apoptotic signaling of PF-68 in MDR cells.

4. Conclusion

Three NLPs containing non-ionic surfactants were prepared to overcome MDR in PTX-resistance A549/T cells. NLPs1 or NLPs3 demonstrated reduced particle size and slower release rate compared with LPs, but NLPs2 showed increased release rate of PTX. A549/T cells treated with NLRs showed an obvious increased level of Rh123 accumulation compared with cells treated with free Rh123 or LRs, which indicated the significant inhibition effects of NLRs on drug efflux. The P-gp detection and ATP determination demonstrated that BNLs could not only interfere P-gp expression on the membrane of drug resistant cells, but also decrease ATP level in the cells. The cytotoxicity, the apoptotic assay and the cell cycle analysis of NLPs against A549/T cells all displayed higher toxicity than LPs. These results suggested that these nanohybrid systems of non-ionic surfactant inserting liposomes could overcome MDR in cancer cells by the combination of drug delivery, P-gp inhibition and ATP depletion, and have potential for treatment of MDR cancer.

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