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Reversal activity of nanostructured lipid carriers loading cytotoxic drug in multi-drug resistant cancer cells

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ABSTRACT

To overcome multi-drug resistance (MDR) of cancer cells, paclitaxel (PTX) and doxorubicin (DOX)-loaded nanostructured lipid carriers (NLC) were prepared by solvent diffusion method using monostearin as solid lipid and oleic acid as liquid lipid matrix. The cytotoxicities and reversal activity of drug-loaded NLC were tested against human breast cancer (MCF-7) cells, human ovarian cancer (SKOV3) cells and their multi-drug resistant (MCF-7/ADR and SKOV3-TR30) cells. The chemical conjugant of folic acid and stearic acid (FA–SA) was further synthesized to prepare folated NLC. Comparing with taxol and doxorubicin solution, the NLC loading PTX exhibited high cytotoxicities in MCF-7 and MCF-7/ADR cells, while the NLC loading DOX only indicated high cytotoxicity in MCF-7/ADR cells. The reversal powers of the NLC loading PTX and DOX were 34.3 and 6.4 folds, respectively. The NLC loading PTX and DOX showed the same trends of enhanced cytotoxicity against SKOV3 and SKOV3-TR30 cells. The reversal powers were 31.3 and 2.2 folds for the NLC loading PTX and DOX, respectively. The modification of NLC with FA–SA could further enhance the cytotoxicities of drug in drug sensitive and drug resistant cells.

1. Introduction

The multi-drug resistance (MDR) is the critical point responsible for most failure of human cancer chemotherapy, which has no selectivity in chemical structure and intracellular target of drug (Ozben, 2006). In spite of the mechanism for MDR is not unveiled completely, avoiding the appearance of drug resistance and modulation of the MDR has been the great challenge to cancer therapy in clinic and laboratory.

Paclitaxel (PTX, an antimicrotubule agent) and doxorubicin (DOX, an anthracycline antibiotic) are typical and commonly used cytotoxic drugs showing obvious anticancer effect to varieties of human cancer in clinic. However, once the MDR occurred, the therapeutic effect was significantly decreased. For occurrence of MDR, a key role was P-glycoprotein (P-gp), which efflux out the chemotherapeutic agents through ATP-dependant transport leading to intracellular deficiency concentration of drug (Zhou et al., 2007). P-gp has been contributed to resistance of natural product-based chemotherapeutics, including taxanes, anthracyclines, vinca alkaloids, podophyllotoxins, and camptothecins (Mechetner et al., 1998). Nanoparticles (NPs) delivery systems were known as the intracellular delivery carries for PTX or DOX (Antonella et al., 2000). A number of studies supposed that intracellular transport of drug-loaded NPs delivery systems via endocytosis was an alternative route of internalization of drug into cells, which may enable bypassing or inhibiting P-gp-mediated efflux (Mahesh et al., 2006; Goren et al., 2000). Sharma et al. demonstrated that administration of PTX entrapped in liposomes, at doses exceeding the maximum tolerated dose (>30 mg/kg), led to a delay in cancer growth using a PTX-resistant mouse xenograft model (Sharma et al., 1993). PTX loaded in novel cetyl alcohol/polysorbate-based nanoparticles could overcome multi-drug resistance in a human colon adenocarcinoma cell (Koziara et al., 2004). Several polymeric carriers have been proposed to overcome the drug resistance, indicating that the copolymer can reduce ATP production in the drug resistant cells, and no noticeable change in ATP production was found in sensitive cells. The low ATP production in the drug resistant cells decreases the P-gp activity. Moreover, the receptor mediated-endocytosis of liposomal drug has also been regarded as a primary method for bypassing P-gp efflux to overcome drug resistance (Lo et al., 2001).

Nanostructured lipid carriers (NLC) composed of a solid lipid matrix with a certain content of liquid lipid is a new generation of solid lipid nanoparticles (SLN) (Zauner et al., 2001). NLC can avoid or minimize the potential problems associated with SLN such as limited drug loading capacity, adjustment of drug release profile

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and potential drug expulsion during storage (Miglietta et al., 2000). The incorporation of liquid lipid to solid lipid leads to massive crystal order disturbance. The resulted matrix of lipid particles shows great imperfections in the crystal lattice and leaves enough space to accommodate drug molecules, thus, leading to improved drug loading capacity (Davda and Labhasetwar, 2002; Gupta et al., 2004).

As a kind of promising nanoparticles drug delivery system, NLC has been studied for some years in our group (Hu et al., 2005, 2006). In our previous studies, the NLC loading hydrophobic drugs could reach higher drug loading comparing with that of SLN, and the controlled drug release was achieved by adjusting the content of liquid lipid. Further, the cellular uptake tests demonstrated the faster internalization of NLC into cancer cells. Cancer cells divide rapidly and need folic acid for DNA synthesis, thus the amount and activity of folic acid receptor in the membrane of cancer cells was higher than that of normal cells. In our previous studies, it was demonstrated that the cellular uptake of lipid nanoparticles was enhanced after the surfaces were modified by folic acid (Yuan et al., 2008).

In the present study, PTX and DOX were chosen as hydrophobic cytotoxic drugs to prepare drug-loaded NLC by solvent diffusion method. The cytotoxicities of NLC loading drug against human cancer cells (human breast cancer MCR-7 cells and human ovarian cancer SKOV3 cells) and their multi-drug resistant variants were investigated to evaluate the reversal activity, comparing with that of taxol and doxorubicin solution. The chemical conjugant of FA–SA was further synthesized to prepare folated NLC, and the cytotoxicities of folated NLC were also studied.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochlorate (DOX·HCl) was gifted from Hisun Pharm. Co. (Zhejiang, China). Paclitaxel was purchased from Zhanwang Biochemical Co. (Huzhou, China). Monostearin (Shanghai Chemical Reagent Co., China) was used as solid lipid material of nanoparticles. Oleic acid (Yixing Chemical Reagent Co., China) was chosen as liquid lipid material for NLC. The surfactant, poloaxmer 188, was provided by Shenyang Pharmaceutical University, Jiqi Co. Ltd., China. Folic acid (FA) was purchased from Acros Organic, USA. Stearic acid (SA) was supplied by Shanghai Chemical Reagent Co. Ltd., China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Trypsin and RPMI 1640 Medium were purchased from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biologic (Hanghzou, China). All other chemicals were analytical or chromatographic grade.

2.2. Synthesis of FA–SA

The conjugant of FA–SA was synthesized referring to the literature (Yuan et al., 2008). Briefly, 20 mg stearic acid (SA) was dissolved into 5 ml dimethylformamide (DMF), and then 20 mg EDC was added into the stearic acid DMF solution. After EDC reacted with SA for 1 h under room temperature, 30 mg folic acid and 0.5 ml pyridine was added into the above reaction solution, and the further reaction was conducted overnight. To precipitate the FA–SA, 20 ml distilled water was added into reaction mixture. The dispersion was dialysed against distilled water by using cellulose membrane (8000 molecular weight cut-off) for 3 days, to remove unreacted folic acid, EDC and the water-soluble side product. The precipitate was collected by filtration with 0.45 μm millipore filter, and lyophilized.

2.3. Preparation of NLC

The doxorubicin base (DOX) was used as model drug to be encapsulated by NLC. The doxorubicin hydrochlorate (DOX·HCl) was stirred with twice mole of TEA in DMSO overnight to obtain the DOX base (Lee et al., 2005).

The NLC were prepared according to previous reports (Hu et al., 2005, 2006). Briefly, 3 mg hydrophobic drug (PTX or DOX), 6 mg OA and 54 mg monostearin were dissolved in 6 ml warm ethanol (70 °C). The resultant organic solution was quickly dispersed into 60 ml distilled water under mechanical stirring (DC-40, Hangzhou Electrical Engineering Instruments, China) with 400 rpm in water bath of 70 °C for 5 min. The obtained pre-emulsion (melted lipid droplet) was then cooled to room temperature till NLC dispersion was obtained.

Blank NLC was also prepared as described above only without drug in organic solution. To prepare folated NLC, 6 mg FA–SA was used instead of the same amount of monostearin.

The pH value of the obtained NLC dispersion was adjusted to 1.20 to form the NLC aggregation by adding 0.1N of hydrochloric acid. Then the NLC precipitate was harvested by centrifugation at 20,000 rpm for 15 min (3K30, Sigma, Germany). Finally, the collected precipitate was re-suspended in distilled water containing 0.1% poloxamer 188 by probe-type ultrasonic treatment with 20 times (200 W, active every 2 s for a 3 s duration) (JY92-II, Scientz Biotechnology Co., Ltd., China).

2.4. Characterization of NLC

The average diameters and zeta potentials of blank or drug-loaded NLC were determined by Zetasizer (3000 HS, Malvern Instruments, UK). Before the determination, the blank or drug-loaded NLC dispersion were diluted 20 times with distilled water.

To determine the drug entrapment efficiency, the obtained NLC dispersion was precipitated by the pH adjustment. After the centrifugation at 20,000 rpm for 15 min (3K30, Sigma, Germany), the NLC precipitate was harvested and the drug content in the supernatant was measured. The collected NLC precipitate was re-dispersed in phosphate buffer solution (PBS, pH 7.2) containing 2 M sodium salicylate (for PTX) or 0.1% (w/v) sodium lauryl sulfate (for DOX) and surged by vortex (XW-80A, Instruments factory of Shanghai Medical University, China) for 3 min to dissolve the surface attached drugs, and treated with centrifugation. Drug content in the supernatant was also measured.

The quantification of PTX was analyzed by HPLC method (Agilent 1100 series, USA), using C18 column (DiamohsilTM 250 mm × 4.6 mm, 5 μm) under 35 °C. AUV detector (Agilent, USA) in set wavelength of 227 nm was used. The mobile phase was a mixture of acetonitrile and water (50:50, v/v) with flow rate of 1.0 ml/min. Injected volume of the sample was 20 μl. The calibration curve of peak area against concentration of paclitaxel was $y = 32.361x - 16.282$ under the concentration of paclitaxel 0.5–120 μg/ml ($R^2 = 0.9994$, where y = peak area and x = paclitaxel concentration), the limit of detection was 0.01 μg/ml.

The DOX content was determined by fluorescence spectrophotometer (F-2500, Hitachi, Japan). The excitation and emission was set at 505 and 560 nm, respectively. The calibration curve of fluorescent intensity against concentration of doxorubicin was $y = 244x + 11.399$ under the concentration of doxorubicin 0.2–10 μg/ml ($R^2 = 0.9993$, where y = fluorescent intensity and x = doxorubicin concentration), the limit of detection was 0.05 μg/ml.

The drug entrapment efficiency (EE) and drug loading (DL) of NLC were calculated from the following equations:

$$\mathrm{EE}(\%) = \frac{W_a - W_{s_1} - W_{s_2}}{W_a} \times 100 \quad (1)$$

$$\mathrm{DL}(\%) = \frac{W_a - W_{s_1} - W_{s_2}}{W_a - W_{s_1} - W_{s_2} + W_L} \times 100 \quad (2)$$

where W_a was the amount of drug added in system, W_{s_1} was the analyzed amount of drug in the supernatant after the first centrifugation, W_{s_2} was the analyzed amount of drug in the supernatant after the second centrifugation. W_L was the weight of lipid added in system.

2.5. In vitro release study

PBS (pH 7.2) containing 2 M sodium salicylate or 0.1% (w/v) sodium lauryl sulfate was used as dissolution medium for PTX and DOX, respectively. After the separated NLC precipitate was dispersed in respective release medium and surged by vortex for 3 min to remove the adsorbed drug on the NLC surface. The NLC was collect by centrifugation, and then re-dispersed in respective release medium. The drug release tests were performed under horizontal shaking (Shellab1227-2E, Shellab, USA) at 37 °C and 60 strokes/min. One milliliter of the dispersion was withdrawn from the system at definite time interval and treated with centrifugation. The drug concentration in the supernatant was measured. The amount of the released drug at each time was then calculated. Each formulation was investigated for three times.

2.6. Cell culture

MCF-7 (human breast cancer cells) and MCF-7/ADR (multi-drug resistant variant) were donated from the first Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China). SKOV3 (human ovarian cancer cells) and SKOV3-TR30 (multi-drug resistant variant) were obtained from Women's Hospital, College of Medicine, Zhejiang University (Hangzhou, China). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 units/ml streptomycin.

2.7. Cytotoxicity assay

Cells were seeded in a 96-well plate at a seeding density of 10,000 cells per well in 0.2 ml of growth medium consisting of RPMI 1640 with 10% FBS and antibiotics. After cells were cultured at 37 °C for 24 h, the growth medium was removed and growth medium containing the different amount of drug (in solution or NLC fomulation) or blank NLC was added. The cells were further incubated for 48 h. Then 100 μl of fresh growth medium containing 50 mg MTT was added to each well and cells were incubated for another 4 h. After removing the unreduced MTT and medium, each well was washed with 100 μl of PBS, and 180 μl of DMSO were then added to each well to dissolve the MTT formazan crystals. Finally, the plates were shaken for 20 min and the absorbance of formazan product was measured at 570 nm in a microplate reader (BioRad, Model 680, USA). Survival percentage was calculated as compared to mock-treated cells (100% survival). All the experiments were performed in triplicate.

2.8. Determination of intracellular drug content

Cells were seeded in a 24-well plate at a seeding density of 100,000 cells per well in 1 ml of growth medium and allowed to attach for 24 h. The cells were then incubated with free drug solution, drug-loaded monostearin NLC and drug-loaded monostearin NLC with 10 wt.% FA–SA (drug concentration: 2 μg/ml) in growth medium for 1, 2, 4, 12, 24 h. After the cells were washed with PBS thrice, 100 μl trypsin PBS solution (2.5 μg/ml) was added. After the further incubation for 5 min, the cells were harvested by adding 400 μl methanol, and was then subjected to probe-type ultrasonic treatment (400 W, 10 cycles with 2 s active-3 s duration, JY92-II, Scientz Biotechnology Co., Ltd., China) in ice bath. The obtain cell lysate was centrifuged at 10,000 rpm for 10 min. The drug content in the supernatant after centrifugation was measured. The protein content in the cell lysate was measured using the micro-BCA protein assay kit. The cellular uptake percentages of drug were calculated from the following equation:

$$\text{Drug uptake percentage } (\%) = \frac{C/M}{C_0/M_0} \times 100 \quad (3)$$

where C was intracellular drug concentration in different time, M was unit weight (milligram) of cellular protein in different time, C_0 was initial drug concentration, M_0 was initial unit weight (milligram) of cellular protein.

3. Results and discussions

3.1. Characteristic of NLC

Blank and drug-loaded NLC were prepared by solvent diffusion method. The volume average diameters, the polydispersity indexes and zeta potentials of resulted NLC are listed in Table 1. Due to the NLC used in the cellular uptake and cytotoxicity tests were the redispersions in 0.1% poloxamer 188 solutions, the results were the data of NLC redispersions.

From Table 1, it was found that the size of drug-loaded NLC was bigger than that of blank NLC due to the incorporation of drug into the NLC matrix, which increased the amount of solid phase and reduced the zeta potential of prepared NLC. The FA–SA modified NLC had relatively smaller particle size than that without modification, which may be caused by the enhanced hydrophilicity of NLC surface due to the incorporation of FA–SA. All of the absolute values for zeta potential of obtained NLC were above 30 mV. This demonstrated that the nanoparticles redispersion was a physically stable system.

Table 1 also indicated the encapsulation efficiency (EE) and drug loading (DL) of drug-loaded NLC. About 70 wt.% EE could be reached by present preparation method, even the NLC were washed with dissolution medium for 3 min. No obvious EE and DL change was observed after the NLC was modified with FA–SA.

3.2. In vitro drug release behaviors

Figs. 1 and 2 show in vitro PTX and DOX release profiles from drug-loaded NLC. After modified with FA–SA, drug-loaded NLC shown no obvious change in drug release profile. PTX presented sustained release profile from PTX-loaded NLC. Only about 70 wt.% PTX was released in 48 h. On the other hand, above 90 wt.% DOX was released from DOX-loaded NLC in 24 h. Because PTX is a kind of highly lipophilic drug with better affinity for lipid material comparing with DOX, the PTX-loaded NLC showed relative slower in vitro release profile than DOX-loaded NLC.

3.3. The anti-cancer and reversal MDR activities of drug-loaded NLC

The 50% cellular growth inhibitions (IC_{50}) of Blank NLC and drug-loaded NLC against MCF-7, MCF-7/Adr, SKOV3 and SKOV3-TR30 are

Table 1
Properties of blank and drug-loaded NLC ($n = 3$)

Formulation	Mean particle diameter (nm)	Polydispersity index (PI)	Zeta potentials (mV)	Encapsulation efficiency (%)	Drug loading (%)
Blank NLC	134.4 ± 21.0	0.361 ± 0.024	−45.5 ± 3.0	–	–
Blank NLC with 10 wt.% FA–SA	117.5 ± 22.8	0.358 ± 0.032	−44.5 ± 3.3	–	–
PTX-loaded NLC	561.7 ± 38.3	0.382 ± 0.039	−37.4 ± 2.8	68.67 ± 4.50	3.42 ± 0.17
PTX-loaded NLC with 10 wt.% FA–SA	501.6 ± 41.9	0.407 ± 0.038	−38.7 ± 2.7	70.65 ± 0.31	3.60 ± 0.01
DOX-loaded NLC	368.9 ± 35.7	0.356 ± 0.019	−33.2 ± 2.2	66.97 ± 0.23	3.24 ± 0.01
DOX-loaded NLC with 10 wt.% FA–SA	306.3 ± 40.1	0.325 ± 0.022	−32.1 ± 2.5	67.05 ± 2.60	3.24 ± 0.12

d_v, PI, ζ, EE and DL indicate the volume average diameter, polydispersity index, zeta potential, drug entrapment efficiency and drug loading, respectively.

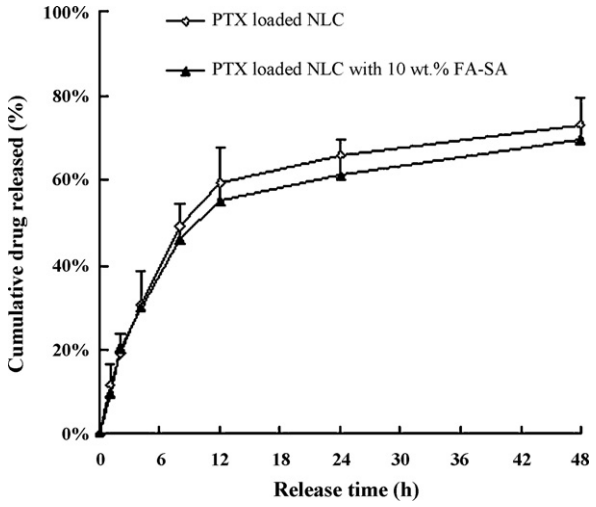

Fig. 1. In vitro paclitaxel release profiles of paclitaxel-loaded NLC.

Table 2
Cytotoxicities of blank NLC, drug solution and drug-loaded NLC against MCF-7 cells and MCF-7/ADR cells

Formulation	IC_{50} (μg ml^{-1})		Reversal power
	MCF-7	MCF-7/ADR	
Blank NLC	455.49 ± 27.68	469.74 ± 30.02	–
Blank NLC with 10 wt.% FA–SA	425.98 ± 21.76	450.21 ± 25.91	–
Taxol	0.290 ± 0.011	8.61 ± 0.28	–
PTX-loaded NLC	0.075 ± 0.010	0.065 ± 0.006	34.3
PTX-loaded NLC with 10 wt.% FA–SA	0.058 ± 0.006	0.033 ± 0.004	52.2
DOX solution	0.176 ± 0.005	6.20 ± 0.22	–
DOX-loaded NLC	0.150 ± 0.007	0.83 ± 0.05	6.4
DOX-loaded NLC with 10 wt.% FA–SA	0.168 ± 0.003	0.71 ± 0.04	8.3

Reversal power was calculated from the equation of $(R_f/R_N)/(S_f/S_N)$. R_f: IC_{50} value of drug solution against drug resistance cells; R_N: IC_{50} value of drug-loaded NLC against drug resistance cells; S_f: IC_{50} value of drug solution against drug sensitive cells; S_N: IC_{50} value of drug-loaded NLC against drug sensitive cells.

shown in Tables 2 and 3. It was clear that the blank NLC before and after FA–SA modification showed very high value of IC_{50} in the two kinds of cell-lines above and their multi-drug resistant variants, suggesting these lipid materials were safe for the use of drug carriers.

In sensitive human breast cancer cells (MCF-7), the encapsulation by NLC significantly enhanced the cytotoxicity of PTX (Table 2), comparing with that of taxol. For the MDR cells (MCF-7/ADR), the IC_{50} value of taxol was nearly 30 folds higher than that in sensitive cells. However, PTX-loaded NLC against MDR cells had lower IC_{50} value, even lower than that in sensitive cells, which meant the PTX-loaded NLC could completely reverse the PTX-resistant of MCF-7/ADR cells. The reversal power of PTX in NLC against MCR-7/ADR was 34.3. After the modification with FA–SA, the reversal power of PTX-loaded NLC against MCF-7/ADR was significantly increased to 52.2, which caused by the enhanced endocytosis mediated by the folate receptor. The increased cellular uptake could bypass or inhibit the pump efflux of P-gp.

Comparing with free DOX solution, DOX-loaded NLC could not clearly improve the cytotoxicity of DOX against MCF-7 cells, even

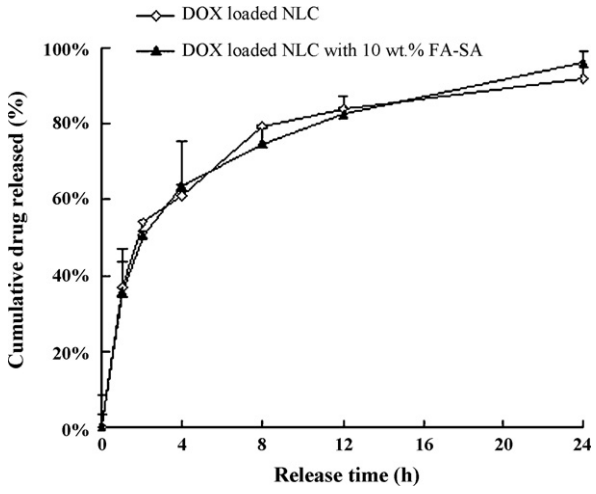

Fig. 2. In vitro doxorubicin release profiles of doxorubicin-loaded NLC.

Table 3
Cytotoxicities of blank NLC, drug solution and drug-loaded NLC against SKOV3 cells and SKOV3-TR30 cells

Formulation	IC_{50} (μg ml^{-1})		Reversal power
	SKOV3	SKOV3-TR30	
Blank NLC	487.92 ± 31.85	498.97 ± 35.97	–
Blank NLC with 10 wt.% FA–SA	431.23 ± 27.43	464.72 ± 29.32	–
Taxol	0.160 ± 0.003	9.35 ± 0.25	–
PTX-loaded NLC	0.053 ± 0.006	0.100 ± 0.015	31.0
PTX-loaded NLC with 10 wt.% FA–SA	0.035 ± 0.005	0.072 ± 0.005	28.4
DOX solution	0.52 ± 0.08	1.83 ± 0.11	–
DOX-loaded NLC	0.33 ± 0.04	0.52 ± 0.09	2.2
DOX-loaded NLC with 10 wt.% FA–SA	0.25 ± 0.04	0.39 ± 0.05	2.3

Reversal power was calculated from the equation of $(R_f/R_N)/(S_f/S_N)$. R_f: IC_{50} value of drug solution against drug resistance cells; R_N: IC_{50} value of drug-loaded NLC against drug resistance cells; S_f: IC_{50} value of drug solution against drug sensitive cells; S_N: IC_{50} value of drug-loaded NLC against drug sensitive cells.

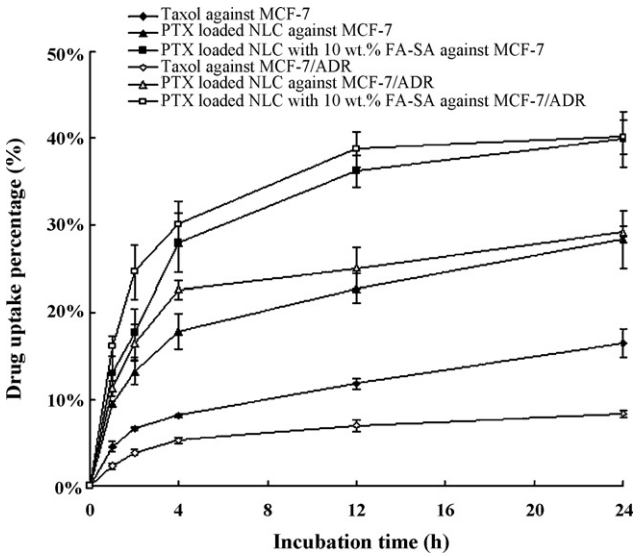

Fig. 3. The drug uptake percentages against incubation time after the MCF-7 and MCF-7/ADR cells were incubated with different formulations of paclitaxel. Mean ± S.D., $n = 3$.

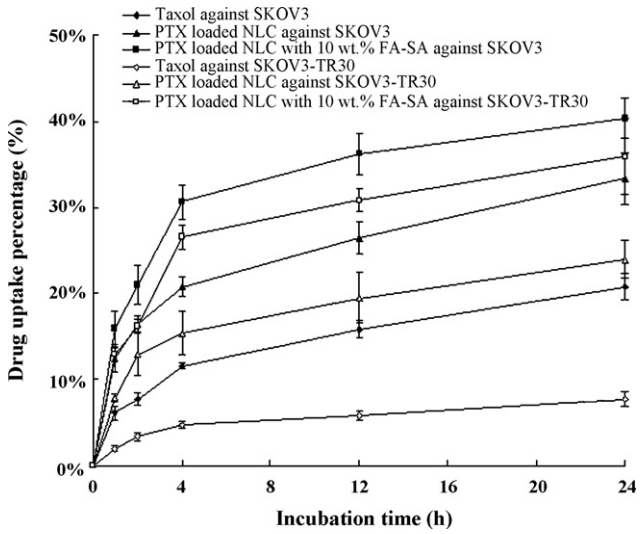

Fig. 5. The drug uptake percentages against incubation time after the SKOV3 and SKOV3-TR30 cells were incubated with different formulations of paclitaxel. Mean ± S.D., $n = 3$.

the NLC was modified with FA–SA (Table 2). The results may due to the burst drug release behavior of DOX-loaded NLC at the initial stage (Fig. 2), which led to the lower DOX concentration internalized into cells. However, after the DOX was encapsulated into NLC, the cytotoxicity of DOX against MCF-7/ADR cells was increased about 7.5 folds. The reversal power of NLC was 6.4. After the modification with FA–SA, the reversal power increased upto 8.3.

In SKOV3 cells and SKOV3-TR30 cells, PTX-loaded NLC and DOX-loaded NLC presented the same trend of enhanced cytotoxicity (Table 3). The PTX-loaded NLC showed significant improvement of cytotoxicity in drug sensitive and resistant cells, with reversal power of 31.0 (Table 3). Formulation of folated NLC showed no improvement in reversal effect because the enhancement of folated NLC loading PTX against sensitive cells (SKOV3) was stronger than that in drug resistant cells (SKOV3-TR30). DOX-loaded NLC with or without FA–SA modification also showed poor improvement of cytotoxicity in both drug sensitive and resistant cells. As a result, the reversal powers of DOX-loaded NLC were below 2.5. The results could also attribute to the burst drug release behavior of DOX-loaded NLC at the initial stage.

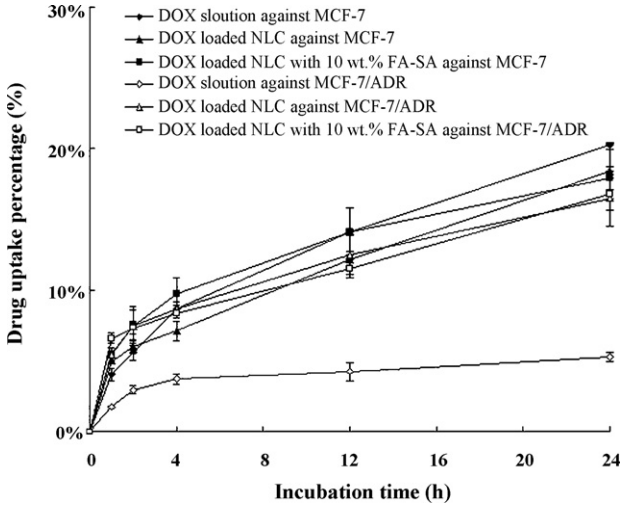

Fig. 4. The drug uptake percentages against incubation time after the MCF-7 and MCF-7/ADR cells were incubated with different formulations of doxorubicin. Mean ± S.D., $n = 3$.

Comparing with free drug solution, the drug loaded in NPs presented improved cytotoxicity against drug sensitive cancer cells. The same results were observed other research (Yuan et al., 2008; Serpea et al., 2004), even in drug resistant cells (Antonella et al., 2000; Nemati et al., 1996) and in vivo (Barraud et al., 2005). Here, NLC as a lipid NPs delivery carrier presented reversal activity in multi-drug resistant cells (MCF-7/ADR and SKOV3-TR30). The addition of FA–SA, a targeting agent to folate receptor widely over-expressed in cancer cell membrane, seemed to be a great helper for drug-loaded NLC to bypass or inhibit the pump efflux of P-gp.

The mechanism of enhanced cytotoxicity of drug-loaded lipid NPs was investigated and discussed in previous papers (Antonella et al., 2000; Serpea et al., 2004). It is obvious that improved cytotoxicity is due to the increased intracellular drug concentration. To our knowledge, internalization of drug into cells was enhanced when the drug was incorporated in NLC, due to the membrane affinity of lipid material and nano-scaled size of NLC.

In drug resistant cells, over-expression of ATP-binding cassette (ABC) transporters, typically P-gp, seems to be responsible for MDR (Ozben, 2006). The published paper (Mechetner et al., 1998) revealed the cellular uptake of drug-loaded NPs was an ATP-mediated action. Thus, NLC was presumed to act as a competitive inhibitor to P-gp's action. The inhibition would be responsible for the increased drug uptake and MDR reversal in multi-drug resistant cells. Based on controlled release profile of drug-loaded NLC within 24 or 48 h, the other probable reason for the reversal of MDR reversion was the accumulation of NLC in cytoplasm, which served as a drug-container releasing the drug continuously to overcome the drug reduction by P-gp efflux.

3.4. Intracellular drug concentration

To clear the relationship between cytotoxicity and the drug concentration in cells, the cellular uptakes of PTX and DOX measurement were conducted. After treating physical mixture of drug-loaded NLC and cells with methanol at 70 °C, the extraction efficiency of drug could reach up to 94.3%.

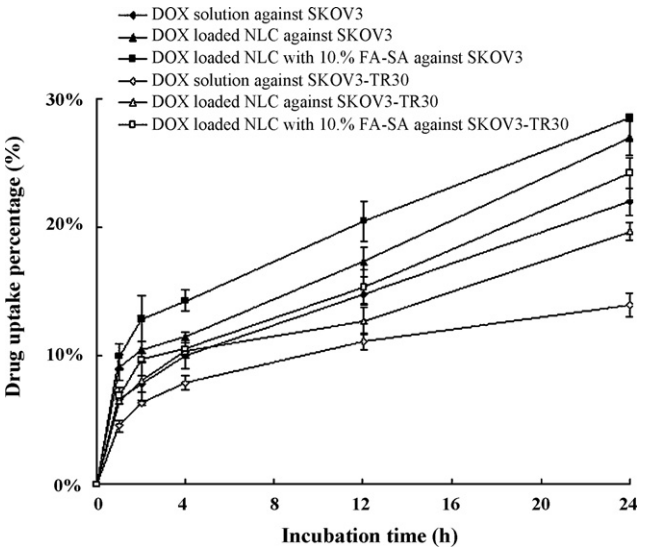

Fig. 6. The drug uptake percentages against incubation time after the SKOV3 and SKOV3-TR30 cells were incubated with different formulations of doxorubicin. Mean ± S.D., $n = 3$.

Fig. 3 shows the cellular uptake percentages of PTX at different incubation time when the MCF-7 and MCF-7/ADR cells were incubated with different PTX formulations. The cellular uptake percentages of PTX delivered by the same NLC formulation in MCF-7/ADR cells were similar or even higher than that in MCF-7 cells at the same incubation time. The result was just consistent with the completely reversal (the cytotoxicity of PTX-loaded NLC in MCF-7/ADR cells was higher than that in MCF-7 cells) of PTX-resistance of MCF-7/ADR cells.

Fig. 4 shows the cellular uptake percentages of DOX at different incubation time when the MCF-7 and MCF-7/ADR cells were incubated with different DOX formulations. Besides the cellular uptake of DOX solution in MCF-7/ADR was lower, no obvious difference was found from the cellular uptakes of DOX in other formulations. The results were consistent with the cytotoxic results of DOX formulations. This means the NLC loading DOX only partially reverse the multi-drug resistance of MCF-7/ADR cells.

Fig. 5 shows the cellular uptake percentages of PTX at different incubation time when the SKOV3 and SKOV3-TR30 cells were incubated with different PTX formulations. The cellular uptake percentages of PTX loaded in NLC obviously increased comparing with that of taxol. However, the cellular uptake percentages of PTX delivered by the same NLC formulation in SKOV3-TR30 cells were still lower than that in SKOV3 cells at the same incubation time. The result could explain the partially reverse activity of NLC loading PTX in SKOV3-TR30 cells.

Fig. 6 shows the cellular uptake percentages of DOX at different incubation time when the SKOV3 and SKOV3-TR30 cells were incubated with DOX of different formulations. It was clear that the cellular uptake percentages of DOX loaded in NLC were higher than that of DOX solution. However, the cellular uptake percentages of DOX delivered by the NLC in SKOV3-TR30 cells were lower than that in SKOV3 cells at the same incubation time. It was the reason that the cytotoxicity of DOX-loaded NLC against SKOV3-TR30 cells was lower than that against SKOV3 cells.

4. Conclusion

Low intracellular drug concentration is the universal character in multi-drug resistant cells when the anticancer agent was administrated. The encapsulation of cytotoxic drug (PTX or DOX) by nanostructured lipid carriers could enhance the cytotoxicity of drug against drug sensitive cells and their multi-drug resistant variant cells, comparing with free drug solution. Lipid matrix-based NLC can increase the drug transport into cancer cells, and overcome the multi-drug resistance. The reversal power in multi-drug resistant cells was improved when the NLC was modified with folic acid, which revealed a potential application for reversal multi-drug resistance human cancer cells.

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