Preparation and cytotoxicity of 2-methoxyestradiol-loaded solid lipid nanoparticles

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The objective of this study was to prepare 2-methoxyestradiol (2-ME)-loaded solid lipid nanoparticles (SLN) by hot homogenization–ultrasonication and evaluate their cytotoxicity on three cell lines, breast cancer [Michigan Cancer Foundation-7 (MCF-7)], prostatic carcinoma (PC-3), and glioma (SK-N-SH), by the sulforhodamineB method. The particle sizes and zeta potentials of the prepared SLN were around 120 nm and −40 mV, respectively. Differential scanning calorimetry (DSC) measurements revealed that the monostearin and 2-ME existed in solid and amorphous states in the SLN prepared, respectively. The high drug entrapment efficiency (>85%) indicated that most 2-ME was incorporated in the SLN. An in-vitro drug release study showed that 2-ME was released from the SLN in a slow but time-dependent manner. The cytotoxicity of 2-ME in SLN on each cell line was significantly enhanced compared with the solution. 2-ME SLN composed of Tween80 was approximately 17-fold more effective on PC-3 cells and 6.7-fold more effective on SK-N-SH cells than in the solution, whereas a lower sensitivity was achieved on MCF-7 cells. In each cell line, the cellular uptake percentages of 2-ME in SLN were much higher than the solution, respectively. In addition, surfactants may exert different effects on the cytotoxicity of 2-ME SLN depending on the cell line. The above assay demonstrated that SLN could significantly enhance the cytotoxicity of 2-ME compared with the free drug because of the increased cellular internalization and concentration of 2-ME. The results suggested that SLN could be an excellent carrier candidate to entrap 2-ME for improving the effectiveness of tumor chemotherapy. *Anti-Cancer Drugs* **23: 185–190 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

2-Methoxyestradiol (2-ME), a metabolite of 17-β-estradiol, functions as an effective anticancer drug against a wide spectrum of solid tumors because of its antiproliferation and antiangiogenic properties [1–3]. 2-ME induces changes in the levels and activities of many proteins involved in the regulation of the cell cycle, including stress kinases, cell division kinases, cyclin B, and regulators of cell cycle arrest and apoptosis [4]. Although 2-ME has shown high potential as an anticancer drug, the therapeutic outcome of its Phase I and Phase II trials in patients with breast cancer, prostate cancer, or other solid tumors remains unsatisfactory because of an unsustained effective drug concentration in the blood and the target site [5–7]. To overcome this problem, sustaining the transport of sufficient drug to the target site and/or enhancing the cytotoxicity of the drug on cancer cells may serve as effective strategies.

In our group, we have recently demonstrated the feasibility of local subcutaneous intratumoral or peritumoral injectable hydrogel loading solid lipid nanoparticles (SLN) encapsulating 2-ME to not only transport 2-ME-loaded SLN directly to the subcutaneous target/tumor site such as the breast or the prostate but also to control their prolonged release for approximately 1–2 months [8–11]. The new preparation can avoid some severe shortcomings of intravenous administration, such as mononuclear phagocyte system (RES) clearance of SLN, systemic distribution, frequent administration, and unsustained effective drug concentration in the target site. Moreover, cancer cells in solid tumors tend to be more resistant to chemotherapy than nonaggregating cancer cells because of various drug permeation barriers, which makes it difficult to achieve high intratumoral drug concentration in solid tumors [12,13]. This type of drug resistance, sometimes referred to as 'noncellular' drug resistance, may further lead to compromised clinical outcomes even though an anticancer drug has strong in-vitro efficacy. We believe that SLN offers a promise to overcome at least some of these obstacles. In addition to the 'noncellular' mechanisms, SLN is expected to enhance the cytotoxicity of 2-ME on subcutaneous tumor cells such as Michigan Cancer Foundation-7 (MCF-7), prostatic carcinoma (PC-3), and SK-N-SH [14–17]. As is known, in the target organ/tissue, the sensitivity of cancer cells to drugs could influence the effectiveness of chemotherapy considerably. The drug incorporated into SLN could be taken up by cancer cells in a way different from the free drug and enhance the sensitivity of cancer cells to

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the anticancer drug to enhance its cytotoxicity [18,19]. These studies point to the possibility that SLN may improve the effectiveness of chemotherapy in cancers, especially those that are comparatively refractory to free drug therapy, such as breast cancer, prostate cancer, and glioma. In addition, SLN could provide other advantages such as physical stability, easy production, excellent biocompatibility, and a possibility of controlled drug release [20].

After demonstrating that local intratumoral or peritumoral injectable hydrogel loading SLN encapsulating 2-ME could directly and mainly transport 2-ME SLN to the target site [8], the specific aim of this study was to investigate the ability of SLN to enhance the cytotoxicity of 2-ME on breast cancer cells, prostate cancer cells, and glioma cells.

To our knowledge, few studies have been conducted to investigate the preparation of 2-ME-loaded SLN and their cytotoxicity until now. The cellular uptake and cytotoxicity of 2-ME SLN composed of different surfactants such as Tween 80 (TSLN) and poloxamer 188 (FSLN) on MCF-7 cells, PC-3 cells, and SK-N-SH cells was evaluated in comparison with 2-ME solution, respectively. In addition, the physicochemical properties of 2-ME loaded SLN, such as particle size, zeta potential, drug entrapment efficiency (EE), drug loading, and in-vitro drug release profiles, were also investigated.

Materials and methods

Materials

2-ME (99.5% in purity) were home-made. Phosphatidylcholine (injection grade) was from Siwei (Zhengzhou, China). Poloxamer188 was supplied by Shenyang Jiqi Pharmaceutical Co. Ltd. (Shenyang, China). Monostearin was from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Compritol 888ATO (ATO888) was purchased from Gattefosse (Saint-Priest, France). Trypsin and RPMI Medium 1640 were from Gibco BRL (Gaithersberg, Maryland, USA). Fetal bovine serum was from Sijiqing Biologic Co. Ltd. (Zhejiang, China). SulforhodamineB (SRB) was from Sigma Co. (St Louis, Missouri, USA). All reagents for high-performance liquid chromatography (HPLC) were of chromatographic purity. Other chemicals used were of analytical grade.

Preparation of 2-ME SLN

SLN were prepared using hot homogenization–ultrasonication. In brief, 20 mg 2-ME was added to 250 mg monostearin and 50 mg ATO888 previously melted at 80°C. Three hundred milligrams of phosphatidylcholine and 200 mg Poloxamer188 (or Tween 80) were dissolved in 10 ml double distilled water and heated to 80°C in a beaker. When a clear homogenous lipid phase was obtained, the hot aqueous surfactant solution was added to the hot lipid phase and homogenization (IKA T25; IKA, Staufen, Germany) was carried out at 10 000 r.p.m. for 2 min at 80°C. The pre-emulsion obtained was probe ultrasonicated (LTD JY92-II, Scientz Biotechnology Co., Ningbo, China) at 100 W for 3 min at 80°C. The nanoemulsion(o/w) obtained was cooled in an ice bath to form FSLN (or TSLN).

Characterization of 2-ME-loaded SLN

The drug-loaded SLN in dispersion were diluted 20 times with distilled water, of which the volume average diameter and zeta potential were determined with a Zeta sizer-Nano-ZS90 (Malvern Instruments, Malvern, UK).

Drug EE was determined by ultrafiltration. The drug EE was calculated from the ratio of the drug amount incorporated into SLN to the total charged drug amount. Ultrafiltration was carried out using Centrisart, which consists of a filter membrane (molecular weight cut off 10 000 Da) between the outer chamber and the sample recovery chamber. Approximately 0.5 ml of SLN dispersion containing approximately 0.8 mg 2-ME was placed in the outer chamber. The unit was centrifuged at 3000 *g* for 15 min. SLN along with the encapsulated drug remained in the outer chamber, and the dispersion medium moved to the sample recovery chamber through the filter membrane. The drug content in the dispersion medium was estimated by HPLC analysis (Agilent 1200 series; Agilent, Palo Alto, California, USA). The chromatographic conditions were as follows: C_{18} column (150 × 4.6 mm, 5 μm) (Agilent), a guard (C_{18}, 10 × 4.6 mm, 5 μm) installed ahead of the analytical column, column temperature 30°C, injection volume 20 μl, mobile phase consisting of methanol and water (65 : 35, v/v) was at a flow rate of 1.0 ml/min, the excitation wavelength and emission wavelength were 285 and 325 nm, respectively. The calibration curve for the quantification for 2-ME was linear over the range of standard concentration between 25 and 1250 ng/ml with a correlation coefficient of $R^2 = 0.9996$. EE and DL of SLN were calculated from the equation $EE = [(W_a - W_s)/W_a] \times 100\%$ and $DL = [(W_a - W_s)/(W_a - W_s + W_L)] \times 100\%$, where W_a is the weight of drug added in the system, W_s the analyzed weight of drug in the dispersion medium after ultracentrifugation, and W_L is the weight of lipid added in the system.

Thermal analysis

DSC analysis was performed to characterize the physical state of the lipid core in TSLN and incorporated 2-ME. TSLN were lyophilized without cryoprotectant to prevent the interruption of the melting transition peak of the cryoprotectant. Samples (8–10 mg) were weighed into an aluminum pan, which was then sealed with a pinhole-pierced cover. Heating curves from 10 to 200°C were recorded with a scan rate of 5°C/min in a DSC (Daojin DSC-60A; Daojin, Kyoto, Japan) equipped with a cooling unit. An empty pan was used as a reference.

In-vitro release study

In-vitro release was performed using the dialysis bag diffusion technique. The dialysis bags (MWCO,

8000–14 000, Sigma) were soaked in deionized water for 12 h before use. The bags containing 1 ml 2-ME SLN suspension were put into a flask containing 20 ml of 0.1 mol/l PBS (pH 7.4) with 1% Tween 80 and 0.02% sodium azide, and then shaken horizontally (ZD-85; Zhejiangjintan, China) at 37°C and 60 strokes/min. One milliliter of the dissolution medium was withdrawn from the system at definite time intervals and centrifuged at 10 000 r.p.m. for 10 min. The supernatant was measured by the HPLC method as described above.

Cytotoxicity

In a 96-well plate, MCF-7, PC-3 cells, and SK-N-SH cells were seeded at a density of 7×10^3 to 8×10^3 cells per well in 0.2 ml of RPMI1640 with 10% fetal bovine serum and antibiotics, and then cultured at 37°C for 24 h. After culture, the growth medium was removed, and the growth medium containing 2-ME dimethyl sulfoxide solution, blank SLN, 2-ME TSLN, and 2-ME FSLN with different concentrations was added, respectively. The cells were further incubated for 48 h. After incubation, cells were fixed by adding 20 µl cold 50% trichloroacetic acid (w/v, 4°C) and incubated at 4°C for 1 h. After the plates had been washed with water and dried in air, an aliquot of 50 µl 4% (w/v) SRB in 1% acetic acid (v/v) was added and stained for 30 min. Excess SRB was removed by washing with 1% acetic acid (v/v) for at least three times, and then a volume of 150 µl Tris base (10 mmol/l) was added to each well and shaken for 15–30 min. The optical density was read (Spectra MR, Dynex, American) at 490 nm. Finally, the values of IC_{50} were determined from concentration-dependent cell viability curves.

Cellular uptake of 2-ME

PC-3, MCF-7, or SK-N-SH cells were seeded in a 24-well plate at a seeding density of 1×10^5 cells per well in 1 ml of growth medium and allowed to attach for 24 h. Cells were then incubated with 2-ME dimethyl sulfoxide solution, 2-ME TSLN (drug concentration: 5 µg/ml) in growth medium for 6, 12, and 24 h. After the cells were washed with PBS, 100 µl trypsin PBS solution (2.5 mg/ml) was added. The cells were further incubated for 5 min. The cells were then harvested by adding 400 µl methanol. The cell lysate was centrifuged at 10 000 r.p.m. 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 the drug were calculated from the following equation [14]:

$$\text{Drug uptake percentage}\,(\%) = \frac{C/M}{C_o/M_o} \times 100$$

where C is the intracellular drug concentration at different times, M the unit weight (milligram) of cellular protein at different times, C_o the initial drug concentration, and M_o the initial unit weight (milligram) of cellular protein.

Statistical analysis

Statistical evaluations were performed by Student's *t*-test of the paired observations to analyze the difference; *P* values lower than 0.05 were considered to indicate significant differences. Data are expressed as means ± SD ($n = 3$).

Results and discussion

Preparation of 2-ME loaded SLN

From Table 1, stable 2-ME-loaded SLN with small particle size (< 200 nm) and high EE (> 85%) were prepared. The SLN with a small mean diameter of approximately 100 nm were excepted. It was demonstrated that smaller particles were more easily taken up by cells [18,21]. In the experiment, it was found that selecting composite lipids (monostearin and ATO888) and increasing their content were beneficial for improving the stability of 2-ME-loaded SLN. The surfactant (Poloxamer188 and Tween 80) had no significant influence on the characteristics and EE of 2-ME SLN.

Physical state of the drug and core lipid

DSC was used to investigate the existing form of 2-ME in SLN such as TSLN. As shown in Fig. 1, the melting endothermic peak of 2-ME was observed at 188.88°C, whereas the thermograms of the lyophilized 2-ME TSLN did not show the endothermic peak for 2-ME, suggesting that most 2-ME was not in crystalline state but in amorphous state [14,22]. In addition, the monostearin bulk showed a main melting peak at 59.65°C, and 2-ME TSLN melted at a comparable temperature of 51.91°C, which showed a main melting transition peak temperature 7°C lower than that of the monostearin bulk, suggesting that the monostearin located in the core of SLN had been successfully solidified.

In-vitro release

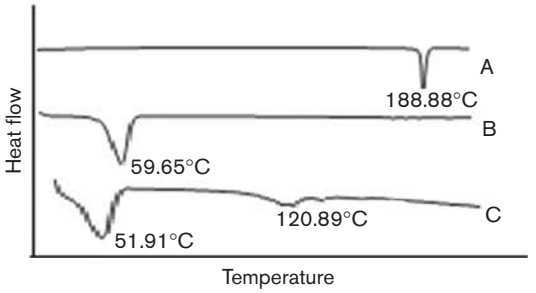
From Fig. 2, propylene glycol solution showed complete release in 12 h; however, an approximately 40% burst drug release was found in the initial 1 h. FSLN and TSLN released nearly 78 and 62% of the drug in 72 h, respectively, without significant burst drug release, indicating that FSLN or TSLN could sustain slower

Table 1 The size, zeta potential, entrapment efficiency (EE), and drug loading (DL) of 2-ME-loaded SLN

	Characters			
Carrier	Size (nm)	Zeta (mV)	EE (%)	DL (%)
2-ME FSLN	135 ± 21	−38.8 ± 2.56	89.12 ± 2.85	2.17 ± 0.17
2-ME TSLN	122 ± 18	−40.3 ± 2.6	88.32 ± 2.07	2.09 ± 0.13

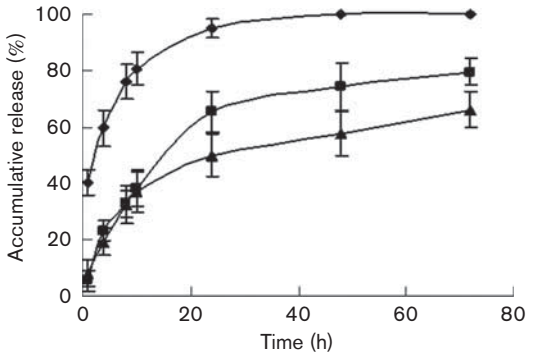
All data are represented as mean ± SD ($n = 3$).
FSLN, SLN composed of poloxamer 188; 2-ME, 2-methoxyestradiol; SLN, solid lipid nanoparticles; TSLN, SLN composed of Tween 80.

Fig. 1



Differential scanning calorimetry curves of 2-ME (A), monostearin (B), and 2-ME TSLN (C). The thermal changes were obtained using a differential scanning calorimeter (daojin DSC-60A, Japan) with a heating rate of 5°C/min. 2-ME, 2-methoxyestradiol; SLN, solid lipid nanoparticles; TSLN, SLN composed of Tween 80.

Fig. 2



In-vitro release profiles of 2-methoxyestradiol from lipid nanoparticles in PBS (pH 7.4) at 37°C. Data were expressed as the mean ± SD ($n=3$). ◆, Propylene glycol solution; ■, FSLN; ▲, TSLN. FSLN, SLN composed of poloxamer 188; SLN, solid lipid nanoparticles; TSLN, SLN composed of Tween 80.

release of 2-ME than the solution. In addition, the release rate of 2-ME from TSLN was significantly slower than FSLN within 72 h, demonstrating that the release rate of 2-ME from SLN could also be related to the surfactant, which may be due to the different affinities between 2-ME and the surfactant. As is known, the release rate of a drug from SLN could be controlled by the lipid material in SLN [14].

Cytotoxicity

The 50% cellular growth inhibitions (IC_{50}) of blank SLN and drug-loaded SLN against MCF-7, PC-3, and SK-N-SH for 48 h are shown in Table 2. It was clear that the blank TSLN and blank FSLN showed a very high value of IC_{50} in the three kinds of cell lines above, suggesting that these lipid materials were safe for use as drug carriers.

Figure 3 showed the cell survival curves of each cell line after exposure to 2-ME solution and 2-ME loaded SLN for 48h, respectively. From Table 2 and Fig. 3, the growth

Table 2 The cytotoxicities after 48 h exposure of MCF-7, SK-N-SH, and PC-3 cells to blank SLN, free 2-ME solution, 2-ME FSLN, and 2-ME TSLN

	Cell lines					
	MCF-7		PC-3		SK-N-SH	
Samples	IC_{50} (μg/ml)	Fold	IC_{50} (μg/ml)	Fold	IC_{50} (μg/ml)	Fold
Free 2-ME	1.31 ± 0.06	1	5.61 ± 0.28	1	1.63 ± 0.09	1
2-ME FSLN	0.62 ± 0.04[a]	2.11	0.76 ± 0.06[a]	7.38	0.33 ± 0.04[a]	4.9
2-ME TSLN	0.51 ± 0.05[a,b]	2.56	0.33 ± 0.03[a,c]	17	0.24 ± 0.03[a,b]	6.8
Blank FSLN	670 ± 54		2710 ± 178		1920 ± 182	
Blank TSLN	420 ± 51		6000 ± 132		2800 ± 147	

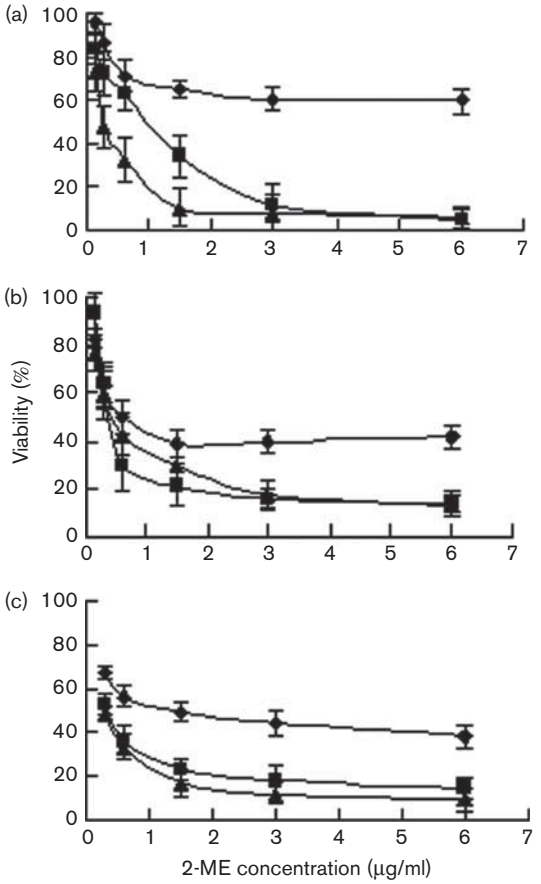
All data was represented as mean ± SD ($n=3$). FSLN, SLN composed of poloxamer 188; MCF-7, Michigan Cancer Foundation-7; 2-ME, 2-methoxyestradiol; PC, prostatic carcinoma; SLN, solid lipid nanoparticles; TSLN, SLN composed of Tween 80.
Statistical significance was assessed by Student's *t*-test of the paired observations.
[a]*P* less than 0.05, in comparison with IC_{50} of 2-ME in solution on MCF-7, PC-3, and SK-N-SH cells, respectively.
[b]*P* greater than 0.05, in comparison with IC_{50} of 2-ME in FSLN on MCF-7 and SK-N-SH cells, respectively.
[c]*P* less than 0.05, in comparison with IC_{50} of 2-ME in FSLN on PC-3 cells.

inhibition of each cell line showed dose-dependence after exposure to 2-ME, either in solution or in SLN, and all of the IC_{50} values of drug-loaded SLN were significantly lower than that of the formulation for drug solution. SLN enhanced cytotoxicities of 2-ME approximately 7.38–17-fold on PC-3 cells and 3.6–6.8-fold on SK-N-SH cells compared with the solution, whereas a lower sensitivity was achieved with 2-ME in SLN on MCF-7 cells. This implies that the drug internalization into cells could be enhanced by the encapsulation of SLN matrix and the endocytosis of SLN, which could be confirmed by other anticancer drugs such as vinorelbine bitartrate, doxorubicin, and paclitaxel [14,15,17].

In the target/tumor site, SLN could enhance the cytotoxicity of 2-ME on PC-3, SK-N-SH, and MCF-7 cells, which could not only decrease the dose and the systemic toxicity but also improve the effectiveness of chemotherapeutics in those cancers, especially PC-3, that are comparatively refractory to drug therapy. For example, none of the concentrations of 2-ME in solution (0.15–6.0 μg/ml) caused 50% inhibition in the growth of PC-3 cells after a 48-h exposure, whereas 2-ME in TSLN strongly enhanced cytotoxicity, with values ranging from approximately 50% at 0.33 μg/ml to nearly 100% at 2.25 μg/ml. Thus, a strong sensitization was achieved with 2-ME-loaded SLN, which appear to decrease cell resistance to the drug.

In addition, few works have been conducted to investigate the effect of surfactants on the cytotoxicity of drug-loaded SLN till now. IC_{50} values of 2-ME in TSLN and FSLN revealed no significant difference ($P > 0.05$) on MCF-7 cells and SK-N-SH cells, but a significant difference was detected on PC-3 cells ($P < 0.05$), indicating that the surfactant possibly influenced the

Fig. 3



Cell survival curves of PC-3 cells (a), MCF-7 cells, (b) or SK-N-SH cells (c) after exposure to 2-ME solution (♦), 2-ME FSLN (■), and 2-ME TSLN (▲) at 48 h (mean ± SD, $n=3$). FSLN, SLN composed of poloxamer 188; MCF-7, Michigan Cancer Foundation-7; 2-ME, 2-methoxyestradiol; PC, prostatic carcinoma SLN, solid lipid nanoparticles; TSLN, SLN composed of Tween 80.

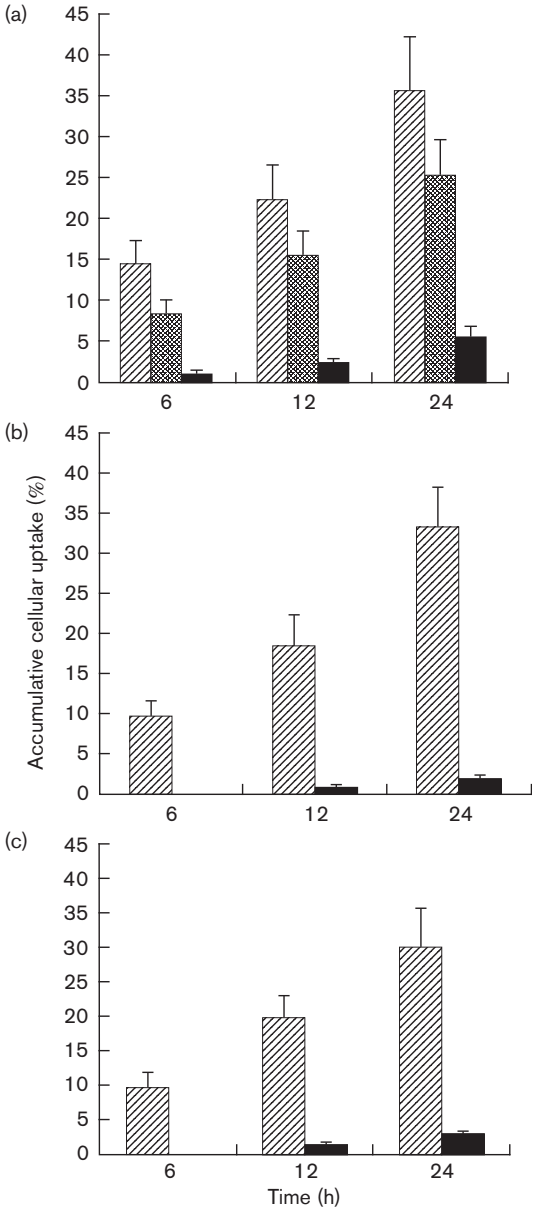
cytotoxicity of 2-ME SLN. The IC_{50} values of 2-ME in TSLN always tended to be lower than that in FSLN, which may be due to the faster release of 2-ME from FSLN than from TSLN, and excessive hydrophilicity of 2-ME FSLN [21]. As is known, the excessive hydrophilicity could prevent the internalization of nanoparticles into cells because of the interaction between the nanoparticles and the lipophilic components of the cell membrane [23].

Cellular uptake of 2-ME

To understand the relationship between cytotoxicity and drug concentration in cells, the cellular uptakes of 2-ME were measured. After the physical mixture of drug-loaded SLN and MCF-7 cells, SK-N-SH cells, or PC-3 cells was treated with methanol at 70°C, the extraction efficiency of the drug could reach up to 91.3, 92.3, or 89.0%, respectively.

Figure 4 showed the cellular uptake percentages of 2-ME at different incubation times when the MCF-7, PC-3, and

Fig. 4



The drug uptake percentage against incubation time after PC-3 (a), MCF-7 (b), or SK-N-SH (c) cells were incubated with 2-ME in solution (■), TSLN (▨), and FSLN (▩) (2-ME: 5 μg/ml) (mean ± SD, $n=3$). A significant difference was found between the cellular uptake percentages of 2-ME in TSLN (or FSLN) and solution in each cell line by Student's *t*-test of the paired observations. FSLN, SLN composed of poloxamer 188; MCF-7, Michigan Cancer Foundation-7; 2-ME, 2-methoxyestradiol; PC, prostatic carcinoma; SLN, solid lipid nanoparticles; TSLN, SLN composed of Tween 80.

SK-N-SH cells were incubated with different 2-ME formulations. From Fig. 4, the cellular uptake percentage of 2-ME in TSLN (FSLN) or solution showed dose-dependence. The intracellular uptake percentage of drug in SLN by each cell line was far higher than in solution at each time interval, which were consistent with the cytotoxic results of 2-ME formulations. It was obvious

that the improved cytotoxicity was due to the increased intracellular drug concentration. To our knowledge, internalization of drug into cells was enhanced when the drug was incorporated in SLN, because of the membrane affinity of the lipid material and nanoscaled size of SLN [15,18,19].

In conclusion, SLN could significantly enhance the cytotoxicity of 2-ME on PC-3 cells, SK-N-SH cells, and MCF-7 cells compared with the free drug because of the increased cellular internalization mediated by the SLN vehicle and the concentration of 2-ME. The surfactant used in SLN possibly influenced the cytotoxicity and cellular uptake of 2-ME, which could be related to the cell line. SLN could be excellent carrier candidates to entrap 2-ME to improve the effectiveness of tumor chemotherapeutics.

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Conflicts of interest

There are no conflicts of interest.

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