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DOX-loaded PEG-PLGA and Pluronic copolymer composite micelles enhances cytotoxicity and the intracellular accumulation of drug in DOX-resistant tumor cells

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In the present study, doxorubicin (DOX) loaded polyethyleneglycol-poly (DL-lactic-co-glycolic acid) micelle as well as composite micelles composed polyethyleneglycol- poly(DL-lactic-co-glycolic acid) (PEG-PLGA) and Pluronic 105 (P105) were constructed. The micelles, with diameter around 106 nm and 85 nm respectively, were prepared by solvent evaporation method. The results showed that the encapsulation of DOX in micelles could significantly enhance its cytotoxicity in a DOX resistant tumor cell line, K562/DOX. The combination of PEG-PLGA and Pluronic further improved both the tumor-suppressive activity and the intracellular accumulation of DOX, indicating that the composite micelles would be potential to reverse the multidrug resistance in tumor cells.

1. Introduction

Chemotherapy resistance, which includes development of simultaneous resistance to multiple drugs often limits the use of chemotherapy (Aroui et al. 2009). One of the most frequent phenotype of multidrug resistance (MDR) is the overexpression of p-glycoprotein (Pgp), a 170-kDa MDR1 gene product (Damiani et al. 2002). The overexpression of Pgp in tumor cells actively efflux out the drug, leading to reduced intracellular drug accumulation and decreased therapeutic efficacy. Therefore, one approach towards increasing drug accumulation in cells is suppressing the efflux mechanism by encapsulating drugs into nanoparticles such as micelles and liposomes.

In recent years, studies have shown that micelles can be used as drug carriers to overcome multidrug resistance of tumors (Chen et al. 2009). Some researches envelopped Pgp substrates in polymeric micelles or nanoparticles to overcome drug resistance (Jabr-Milane et al. 2008). Many amphiphilic agents have been found to enhance the cellular accumulation of anticancer drugs leading to sensitization of Pgp over-expressing cells, such as the Pluronic family composed of poly(ethylene oxide)-(polypropylene oxide)-poly(ethylene oxide) triblock copolymers (Kabanov et al. 2002). Among them, Pluronic 85 (P85) has been extensively studied for its remarkable effect to reverse MDR. It was found that P85 micelles could interact with MDR cancer cells resulting in drastic sensitization of these tumors (Minko et al. 2005). However if P105 as a new member of the same pluronic family, can also induce or enhance the reverse effect on MDR cells still remains unclear.

Doxerubitin (DOX) is one of the anthracycline antibiotics, which play an important role in the treatment of leukaemia. However, multidrug resistance of leukaemia cells decreased its effects largely (Chen et al. 2007). Extensive studies had been carried out to overcome MDR (Aroui et al. 2009; Lukyanova et al. 2009). In the present study, we prepared the drug-carrying composite micelles and demonstrated that a formulation containing the block copolymer PEG-PLGA, P105 and DOX, induced significant cytotoxicity and intracellular accumulation in the DOX resistant human leukemia cell line, K562/DOX.

2. Investigations, results and discussion

As seen from the TEM images (Fig. 1) the copolymers formed spherical particles with narrow diameter distribution. Dynamic light scattering (DLS) studies indicated that the mean diameter of the PEG-PLGA micelle is around 106 nm and the composite micelle is 85 nm. The relative smaller particle size of the composite micelles may due to the lower molecular weight and the more hydrophilic characteristics of P105. The diameter of micelles decreased with the increasing of the hydrophilic chain ratio. The hydrophilic chain of the P105 on the micelle surface induced a much lower critical micelle concentration (CMC) and could improve the stability of the micelles (Xu et al. 2004). Doxorubicin was encapsulated in the hydrophobic segment of the copolymer. Entrapment efficiency reached 39.1 + 2.1% and 45.7 + 2.6%. The relatively low entrapment efficiency of the micelles produced by the aqueous based solvent evaporation technique was probably due to the slow leakage of DOX into the dispersion medium during the stirring procedure (Shi et al. 2008).

Fig. 2(a) compares the cytotoxic effect of free DOX, PEG-PLGA micelles, and composite micelles in K562/MDR cells. PEG-PLGA micelles showed a higher cytotoxicity than free

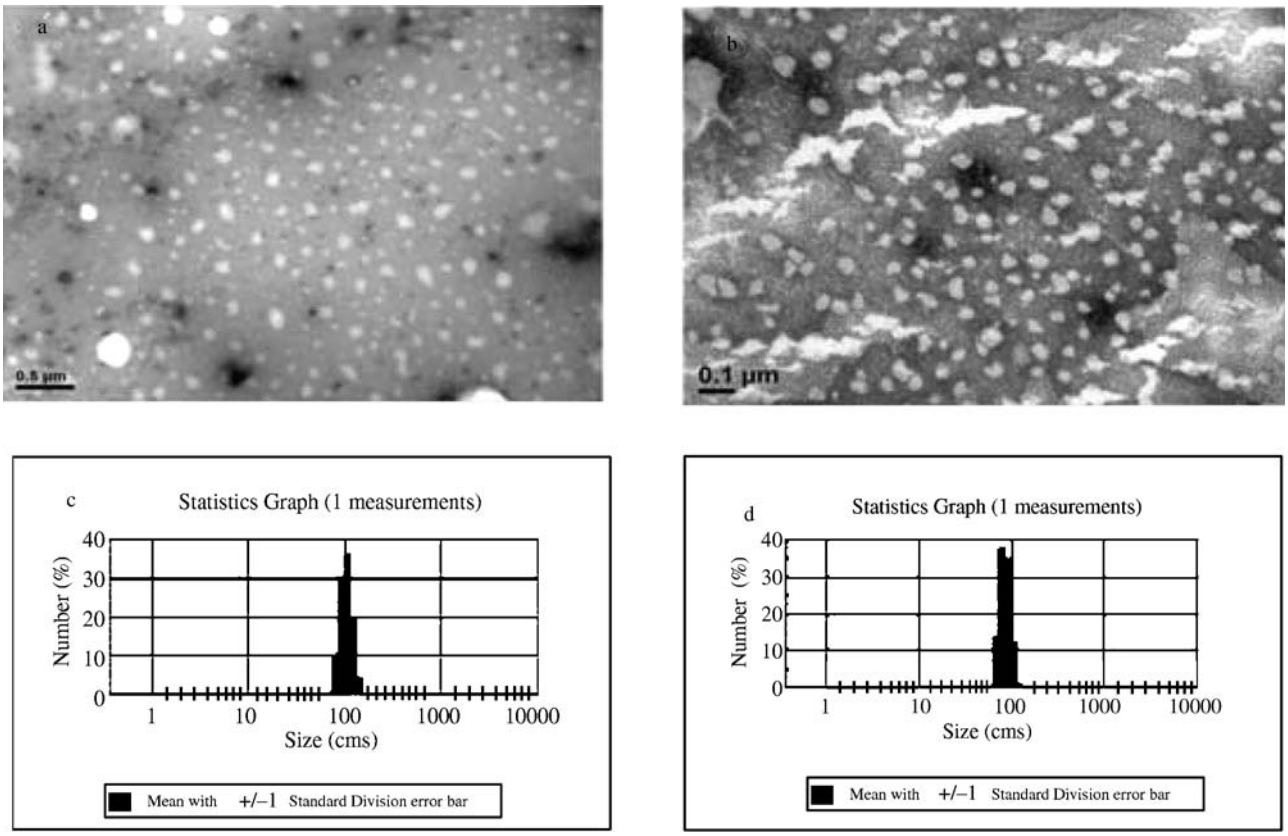

Fig. 1: (a) TEM image of DOX loaded PEG-PLGA micelle; (b) TEM image of DOX loaded PEG-PLGA and P105 composite micelle; (c) Size distribution of DOX loaded PEG-PLGA micelle (d) Size distribution of DOX loaded PEG-PLGA and P105 composite micelle

DOX. While adding P105 into the delivery system, it showed the highest cytotoxicity, indicating the synergistic effect between these two copolymers. The internalization of micellar DOX and free DOX into K562/DOX cells was examined by fluorescent HPLC. As shown in Fig. 2(b), after 2 h of incubation, encapsulation of DOX in micelles significantly enhanced the cellular accumulation of drug. The results correspond with the cytotoxic results very well. The composite micelles showed the highest accumulation, while the free drug was the lowest. Blank composite micelles caused cell death scarcely, which indicated that the cytotoxicity of the micelles resulted from DOX component rather than the carrier material (PEG-PLGA and P105).

Here, we propose that for free DOX, a multi-drug resistant effect, out-fluxing DOX through the p-glycoprotein pump, might play an additional role in decreasing the intracellular concentration of DOX. DOX transported by the micelles enters the cells most likely via internalization, and intracellular entry of DOX-loaded micelles followed by entrapment in endosome/lysosome renders the drug inaccessible for P-gp, then the DOX will be released from the micelle particles inside the cells, and eventually enters the nuclei where DOX is known to exert its cytotoxicity during DNA synthesis. Similar results were also obtained by Shuai et al. (2004). Their studies showed that the patterns of cellular distribution of DOX-loaded MPEG-PCL micelles and free DOX are very different, that is DOX micelles transport into K562/DOX cells by endocytosis, while free DOX enters the cells by simple diffusion.

The highest cytotoxicity observed in the composite micelles may be related to the effects of P105 on the Pgp drug efflux transport system. A lot of studies have shown that Pluronic could inhibit the Pgp function. Similar effects have been observed with other nonionic surfactants such as Cremophor EL, Triton X-100, and so on (Dudeja et al. 1995). There is overwhelming evidence to support inhibition of Pgp by Pluronic block copolymers. This is supported by the observation that the intracellular accumulation of doxorubicin in resistant cancer cells expressing Pgp can be greatly enhanced by treatment with Pluronic. The copolymers affect specifically the Pgp-controlled transport route in MDR cells (Venne et al. 1996). It is also reported that Pluronic block copolymers have a 'double-punch' effect in MDR cells: through ATP depletion and membrane fluidization, a combination which leads to potent inhibition of Pgp (Batrakova et al. 2001a).

Therefore, the higher cytotoxicity of the composite micelles can be attributed to the synergistic effect of the internalization resulted from PEG-PLGA and the inhibition of Pgp caused by P105. As a greater amount of DOX could be intracellularly delivered into cells, the cells were more vulnerable to the cytotoxic effect of DOX.

3. Experimental

3.1. Materials

Doxorubicin (DOX) hydrochloride was obtained from Zhejiang Hisun Pharmaceutical Co, Ltd. MPEGs and ε-Caprolactone was provided by Aldrich. PEG-PLGA and Pluronic 105 were purchased from Shandong Medical Instrumental Institute (China) and sigma (Germany) respectively. The Pgp over-expressing human leukemia cancer cells line K562/DOX were generous gifts from 2nd Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China), and were maintained in RPMI 1640 medium with 10% FBS and 1 μg/ml DOX at 37 °C in a humidified atmosphere with 5% CO_2. MTT was purchased from shanghai Sangon Biological Engineering Technology & Service Co., Ltd. Chloroform (CHCL3), triethylamine (TEN), and other reagents were of analytical grade supplied by Huadong Medical (China).

3.2. Procedures

3.2.1. Preparation and characterization DOX loaded micelles

Micelles were prepared by solvent evaporation method. Briefly, DOX hydrochloride (3 mg) were dissolved in $CHCl_3$ (2 ml) in the presence of triple molar ratio of TEN and stirred (500 rpm) on a magnetic stirrer for 4 h under room temperature. Then PEG-PLGA copolymer (10 mg) with

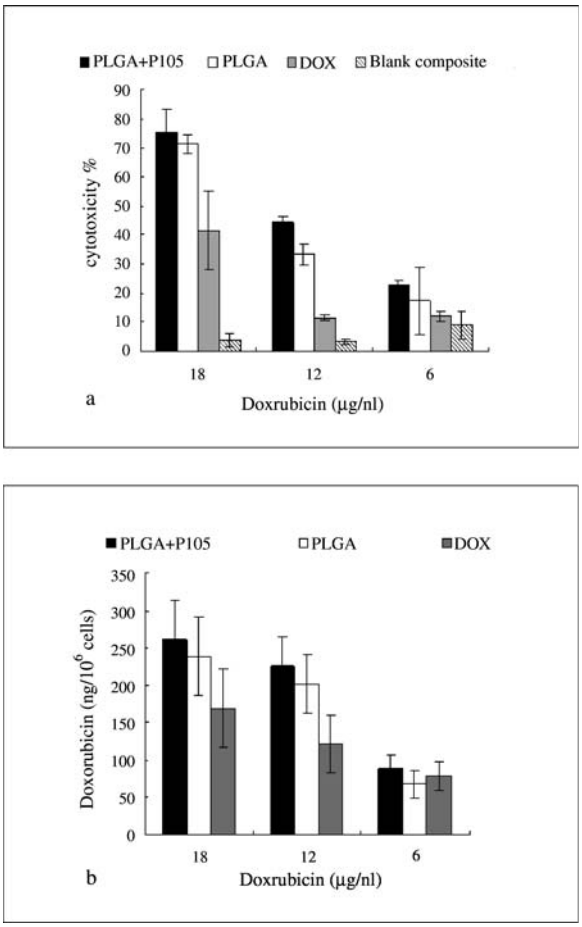

Fig. 2: (a) Cytotoxicity determined by MTT assay in K562/DOX cells. Cells were incubated with different formulation for 48 hours. DOX concentration is varied. Error bars represent the standard deviations (n = 3). (b) The intracellular DOX amount in K562/DOX after incubation with different concentrations of DOX formulation for 2 h

or without P105 (5 mg) was added into the solvent and vortex until it totally dissolved. Afterwards, the organic solution were added into 20 ml distilled water under vigorous ultrasonication agitation. The beaker was stirred vigorously (10,000 rpm) for 1 h, and then slowly stirred (300 rpm) overnight allowing slow evaporation of $CHCl_3$ and formation of the micelles. The residual $CHCl_3$ was completely removed by vacuum distillation with a rotary evaporator. The micelle solution concentrated to 5 ml was filtered with a syringe filter (pore size: 0.22 μm) to eliminate the polymer and DOX aggregates. All procedures were carried out under light protection. The morphologies of the micelles were studied with transmission electron microscopy (TEM; Morgagni 268 D, Fei, The Netherlands) and dynamic light scattering (MALVERN Nano ZS®, Malvern, UK) was used to determine the diameters. Drug entrapment efficiency was investigated by ultracentrifugation, using ultrafiltration membrane (MILIPORE, USA), measured by high performance liquid chromatograph (HPLC, Agilent 1100 system).

3.2.2. Cytotoxicity assay

The cells were cultured for two weeks in drug-free medium prior to their use in the experiments. Then the cells were seeded in 96-well plates at a density of 10^4 cells/well and allowed to grow overnight. A specified concentration of free DOX, DOX loaded micelle was added into the culture medium in the following day, then cultivated for 48 h at 37 °C. The cytotoxic activity of DOX was then evaluated using a standard MTT assay. All the experiments were performed in triplicate.

3.2.3. Cellular uptake determination

Cells suspension, containing different concentration of DOX solutions or DOX loaded micelle, was seeded at a density of 2×10^6 cells/ml in 2 ml of growth medium. The plates were incubated for two hours. Then washed twice with ice-cold PBS to remove un-internalized DOX, and re-suspended in water (1 ml) and treated by probe-type ultrasonication for 20 times (4 °C, 200 W, active every 2 s for a 5 s duration) to obtain the cell lysate. The lysate was centrifuged at 10,000 rpm for 5 min in a microcentrifuge (Eppendorf AG, 22331 Hamburg) to remove cell debris. The supernatant was analyzed using HPLC(Waters 474) equipped with 2475 Multi-Wavelength Fluorescence Detector (Ex(λ) −488 nm/Em (λ) +555 nm).

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