Original Article
Doxorubicin-loaded Pluronic P123-PEG2000-DSPE micelles reverse the drug resistance of breast cancer cells

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Abstract: A new mixed nano-micelle, doxorubicine (Dox)-loaded Pluronic P123/poly (ethylene glycol) 2000-distearoylphosphatidylethanolamine nanomicelles mixed micelles (P123-PEG2000-DSPE (Dox)) were developed to investigate the reversal effects of nanoformulations on breast carcinoma (BC) multidrug resistance (MDR). This study aimed to explore the reversal effects of nanoformulations on BC multidrug resistance. The P123-PEG2000-DSPE (Dox) mixed micelles were prepared and then characterized by the physicochemical parameters with the dynamic light scattering method, drug release profile, and antineoplastic activity including dynamic light scattering method, MTT, immunofluorescence, Western blot and Annexin V-PI in BC MCR-7 cells and MCF-7R, a BC drug resistant cell line. P123-PEG2000-DSPE (Dox) reversed drug resistance with a better effect than PEG2000-DSPE (Dox) in the cells via suppressing the expression of MDR1 and p-gp, decreasing drug efflux, and increasing cellular endocytosis. Furthermore, for the Dox-loaded groups, the cytotoxicity of P123-PEG2000-DSPE (Dox) was better than that of PEG2000-DSPE (Dox) and Dox. PEG2000-DSPE and P123-PEG2000-DSPE, the empty drug carriers, had no cytotoxicity. These findings indicated that P123-PEG2000-DSPE (Dox) micelles could effectively reverse drug resistance in BC cells, which is a promising antitumor drug delivery system.

Keywords: DSPE-PEG2000, Pluronic P123, micelles, breast cancer, drug resistance

Introduction

Multidrug resistance (MDR) is a key way to protect tumor cells from or reduce chemotherapy drugs’ attack, and it also hinders the effectiveness of chemotherapy on tumors [1]. Therefore, relieving the drug resistance of tumor cells and enhancing the toxicity of anti-tumor drugs are important issues to be urgently solved in tumor treatment. Currently, the mechanisms of tumors acquiring MDR are considered to be multifactorial and complex [2], so simultaneously targeting these mechanisms may be an effective way to overcome MDR. The formation mechanism of MDR is complex, and the possible mechanisms include the following aspects: drug intake decreases, efflux increases, DNA repair mechanism enhancement, changes in drug metabolism pathways mutations in drug targets, and apoptosis pathway inactivation or anti-apoptosis defense system over-activation [2, 3]. Among them, decreased drug uptake and increased effusion are the main causes of MDR in tumor cells, mainly caused by the over-expression of the membrane transporter family of ABC (ATP binding cassette), which also includes P-glycoprotein (p-gp) [4, 5]. These proteins, are ATP-dependent and can pump a variety of differently structured drugs out of the cells; significantly reduce the accumulation of anti-cancer drugs in cells, thus developing resistance. In response to clinical multidrug resistance, the strategy is mainly to combine anti-tumor drugs with MDR reversal agents to promote the sensitivity of cancer cells to chemotherapeutic agents, so as to achieve the goal of overcoming MDR [6, 7].

In recent years, nano drug delivery systems have attracted much attention due to their unique physical and chemical properties [8]. Studies have shown that even without the help
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of drug efflux pump inhibitors, nano drug delivery systems can still overcome MDR by endocytosis or phagocytosis to escape the drug efflux protein pump [9-11]. Among them, polymer micelles with the advantage of simple preparation can improve drug distribution and prolong drug cycle time. They have been successfully applied to overcome tumor MDR and promote the toxicity of chemotherapeutic agents [12]. Furthermore, polymer micelles are a supramolecular ordered aggregation of amphiphilic block copolymers spontaneously formed by hydrophobic forces in aqueous solutions. Additionally, it has a special core-shell structure with a hydrophobic block at the core and a hydrophilic block as the shell [13-15]. The polymer micelle delivery system is very popular because of its rich and varied sources and simple preparation process. Pluronic 123 and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethyleneglycol) 2000] (PEG2000-DSPE) are two commonly used antitumor nano-drug delivery systems. Pluronic P123 (hydrophilic-lipophilic balance <20, hydrophobic chain >60) can effectively inhibit p-gp efflux in reducing the microscopic viscosity of the membrane, interfering with the activity of ATPase, and decreasing the production of ATP. Additionally, Pluronic P123 is less than 15,000 Da in molecular weight and its monomer can be excreted by the kidney. PEG2000-DSPE has been used as a common drug carrier against drug resistance and cell invasion. It is an amphiphilic linear polymer with a low CMC and can still form micelles at a lower concentration [16], and the PEG fragments of PEG2000-DSPE can prolong the cycle time of micelles. The reversal effect of mixed micelles of Pluronic-P123 and PEG2000-DSPE for delivery of doxorubicin (Dox) on tumor cell MDR is unclear.

In this study, Dox-loaded Pluronic P123 and PEG2000-DSPE mixed micelles were prepared successfully, and the physicochemical parameters, drug release profile, and antineoplastic activity of the micelles were systematically investigated in a breast cancer (BC) drug resistant cell line (MCF-7R).

Materials and methods

Preparation of nanomicelles

To prepare Dox-loaded PEG2000-DSPE micelles (DSPE-PEG2000 (Dox)), Dox hydrochloride was dissolved in 1 ml of anhydrous methanol and triethylamine (the mass ratio of doxorubicin to triethylamine was 1:2) and was added to obtain hydrophobic Dox. Then, the above Dox solution was mixed with 3 ml of DSPE-PEG2000 solution dissolved in chloroform (the ratio of the lipid material to Dox was 1:5), and the mixed solution of doxorubicin and DSPE-PEG2000 was transferred to a bottle. Organic solvents were removed by vacuum rotary evaporator, so as to form a dry lipid film at the bottom of the bottle. Then the lipid film was dissolved in 2 ml of PBS buffer and hydrated in a water bath at 60°C for 30 minutes. The hydrated solution was filtered through a 0.2 micron pore size polycarbonate membrane to remove uncoated hydrophobic Dox to obtain DSPE-PEG2000 (Dox) solution. To prepare P123-PEG2000-DSPE (Dox), we used P123-PEG2000-DSPE mixed lipid material (1:4 w/w) instead of DSPE-PEG2000 lipid. Additionally, the empty micelles, including PEG2000-DSPE and P123-PEG2000-DSPE, were prepared without Dox.

Encapsulation rate is an important index to evaluate the quality of liposome preparation [17]. In the present study, drug loading and encapsulation efficiency of drug-loaded nanomicelles were determined. The concentration of doxorubicin in the drug-loaded nanomicelles was set to 485 nm and the emission wavelength to 592 nm. The drug loading (EE) and encapsulation efficiency (LC) of nanomicelles were calculated as follows: EE% = Wt/Ws×100% and LC% = Wt/Wo×100%. (Note: Wt: the quality of doxorubicin contained in the nanoparticles; Wo: the initial dose of doxorubicin, and Ws: the total mass of the nanoparticles after lyophilization). Further, the hydrodynamic diameters and distribution of PEG2000-DSPE (Dox) and DSPE-PEG2000 (Dox) were measured using a dynamic light scattering (DLS) instrument under laser wavelength of 633 nm and angle of 90° at 25°C.

In vitro drug release analysis

A total of 200 μL of PEG2000-DSPE (Dox) and P123-PEG2000-DSPE (Dox) solution (1 mg/ml) were put in a dialysis bag (molecular retention of 8,000-12,000 Da), and the dialysis bag was submerged in 35 ml of PBS (pH 7.4 and 5.0, respectively). The entire dialysis system was
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shaken in a constant temperature shaker at 37°C in the dark at an oscillation speed of 200 rpm. Then, 1 ml of dialysate was taken at 0.5 h, 1 h, 2 h, 4 h, 6 h, 9 h, 12 h, and 24 h, respectively, and the same volume of fresh buffer solution was replenished immediately. Finally, the concentration of doxorubicin in the dialysate solution was determined by fluorescence spectrophotometer, and the in vitro release profile of doxorubicin in nanomicelles at different pH values was calculated and obtained.

**Immunofluorescence and laser confocal microscopy**

The MCF-7 and MCF-7R cells were stained according to the instructions of p-gp immunohistochemical detection kit (NO. E670006, Sangon Biotech, Shanghai, China). The p-gp primary antibody was purchased from Abcam (ab103477, Abcam, MA, USA, 1:1000). The MCF-7R endocytosis of Dox, PEG2000-DSPE (Dox), and P123-PEG2000-DSPE (Dox) micelles was measured by a laser scanning confocal microscope.

**MTT assay**

BC cells (MCF-7) and a BC drug resistant cell line (MCF-7R) obtained from Procell Inc (Procell, Wuhan, China) were cultured. When the cells of each group were cultured to logarithmic growth phase, the cell suspension was digested with trypsin and then seeded into 24-well plates at 5×10^3 cells per well. After being mixed with 120 mg/ml MTT for 120 h, the cells were incubated with MTT for 4 h at 37°C under 5% CO₂, and then mixed with DMSO for 30 min. The optical density (OD) at 570 nm was detected by a 96-well microplate reader (DNM-9606, Beijing Prang).

**RT-PCR**

The total RNA of cells in the Dox, PEG2000-DSPE, P123-PEG2000-DSPE, PEG2000-DSPE (Dox), and P123-PEG2000-DSPE (Dox) groups was extracted according to the TRIzol reagent specification, and the reverse mRNA and quantitative PCR kits were used to reverse and quantitatively detect the related mRNA expression. The reaction conditions were as follows: pre-denaturation at 94°C for 4 min, followed by 45 cycles of denaturation at 94°C for 0.5 min, annealing at 60°C for 2 min, and extension at 75°C for 1 min. After the cycle was completed, set 95°C for 20 s, 60°C for 2 min, and 95°C for 20 s. Each sample was equipped with 4 subwells, and the experiment was repeated 3 times. At the same time, a negative control was used to detect the presence of PCR contamination in the reaction system and to exclude primer-dimer interference. The changes in the expression level of the MDR1 mRNA in each group of cells were analyzed based on the collected fluorescence signal values. The primers of MDR1 are forward 5’-TGACATTTATCATAAGGGA-3’ and reverse 5’-TAGACACTTTAGCAACATTTCAA-3’; the primers of GAPDH are forward 5’-AATCCCATCACCACCTTCCA-3’ and reverse 5’-CCTGCTTCACCACCTTCTTG-3’.

**Western blot**

Each group of cells was washed with pre-cooled PBS 1-3 times, and RIPA lysate was added on an ice bath for 30 min, and the cells were shaken once every 10 min. The cells were scraped off with a cell scraper and transferred to a 1.5 ml EP tube. The protein was separated by centrifugation at 12,000 r/min and 4°C for 30 min. The supernatant was removed and the protein was quantified using a BCA protein quantification kit. Each 100 mg of protein was mixed with 1× SDS loading buffer (1:1), and the denatured protein was boiled for 10 min. After 12% SDS-PAGE electrophoresis, the above proteins were transferred to a PVDF membrane, and the p-gp protein bands were detected after antibody incubation.

**Apoptosis assay**

The cells in the logarithmic growth phase of each group were resuspended, and 50,000-100,000 cells were taken and centrifuged at 1000 g for 5 min. The supernatant was discarded. Then, 195 ml of Annexin V-FITC binding solution was added to resuspend the cells, and the cells were stained according to the instructions of the apoptosis detection kit. Apoptosis was detected by a post-flow cytometer. The results consisted of four quadrants, including normal cells [An(-)/Pl(-), at the lower left quadrant], early apoptotic cells [An(+)/Pl(-), at the lower right], late apoptosis and necrosis cells [An(+)/Pl(+), at the upper right], and the damaged cells [(An(-)/Pl(+), at the upper left], and the apoptosis rate was calculated as the ratio of early apoptosis and the proportion of late apoptotic cells to the total cells.
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Statistical analysis
GraphPad Prism 6.05 was employed for statistical analysis of experimental data, and one-way ANOVA and Student’s t test were used for comparative analysis of differences. P<0.05 indicated a significant difference.

Results
Physicochemical property and drug release kinetics of P123-PEG2000-DSPE (Dox) micelles
The particle size parameters of P123-PEG2000-DSPE (Dox) were measured by DLS. Results showed that the hydrodynamic size of P123-PEG2000-DSPE (Dox) was 29±0.3 nm (Figure 1).

Drug release was measured due to its importance for drug delivery systems [18, 19]. Therefore, the drug release profile of doxorubicin nanomicelles at different pH values was simulated in vitro by dialysis. The results showed that drug release from PEG2000-DSPE (Dox) or P123-PEG2000-DSPE (Dox) was slower at pH 7.4 (24% and 25%) than that at pH 5.0 (78% and 78%) (Figure 2).

Cytotoxicity studies of P123-PEG2000-DSPE (Dox)
To evaluate the proliferative potential of cells after being treated with P123-PEG2000-DSPE (Dox), we chose two human BC cell lines, MCF-7 and MCF-7R. It turned out that p-gp was lowly expressed in MCF-7, but was remarkably up-regulated in MCF-7R cells (Figure 3A). Next, MCF-7 and MCF-7R cells were incubated with Dox, PEG2000-DSPE (Dox), or P123-PEG2000-DSPE (Dox) and the cell viability was then evaluated. Compared with Dox and PEG2000-DSPE (Dox), the viability in P123-PEG2000-DSPE (Dox) group was much lower in both MCF-7 and MCF-7R cells (Figure 3B and 3C). Additionally, the cell viability in P123-PEG2000-DSPE (Dox) at Dox concentrations of 0.1-10 μg/ml was lower than that in PEG2000-DSPE (Dox) (Figure 3C).

In addition, the cytotoxicity of the empty P123-PEG2000-DSPE micelles was measured. The results showed that empty P123-DSPE-PEG2000 micelles at 0.05 to 500 μg/ml had almost no toxicity to both MCF-7 and MCF-7R cells (Figure 3D).

Cellular endocytosis of PEG2000-DSPE (Dox) and P123-PEG2000-DSPE (Dox)
We investigated the cellular endocytosis of PEG2000-DSPE (Dox) and P123-PEG2000-
Figure 3. Cytotoxicity of Dox, DSPE-PEG2000 (Dox) and P123-DSPE-PEG2000 (Dox) on MCF-7 and MCF-7R cells. A: p-gp expression in MCF-7 or MCF-7R cells measured by cell immunofluorescence and Western blot; B and C: The toxicity of Dox, DSPE-PEG2000 (Dox) and P123-DSPE-PEG2000 (Dox) on MCF-7 and MCF-7R cells tested by MTT assay; D: The viability of MCF-7 and MCF-7R cells tested by MTT assay.
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DSPE (Dox) micelles by MCF-7R cells. Cells were incubated with Dox, PEG2000-DSPE (Dox), and P123-PEG2000-DSPE (Dox) at 37°C for 0.5 or 1 h with the Dox concentration at 10 μg/ml. The P123-PEG2000-DSPE (Dox) enhanced the cellular endocytosis compared with Dox and PEG2000-DSPE (Dox) (Figure 4).

In addition, the intracellular fluorescence intensity increased when the treatment time was extended from 0.5 to 1 h. The mean intensity of P123-PEG2000-DSPE (Dox) was 2.7-fold higher than that of Dox, and 1.7-fold higher than that of PEG2000-DSPE (Dox) after treatment for 0.5 h, indicating that P123-PEG2000-DSPE (Dox) improved the target efficacy. These results indicated that cellular endocytosis in P123-PEG2000-DSPE (Dox) was much higher than in Dox.

Effect of P123-PEG2000-DSPE (Dox) on p-gp and MDR1

The expression of p-gp in MCF-7R cells after being treated with Dox, PEG2000-DSPE (Dox), P123-PEG2000-DSPE (Dox), PEG2000-DSPE and P123-PEG2000-DSPE was measured by immunofluorescence using laser confocal scanning microscopy. The results showed that the fluorescence intensity of p-gp significantly decreased in P123-PEG2000-DSPE (Dox) and P123-PEG2000-DSPE, compared with Dox, PEG2000-DSPE (Dox), and PEG2000-DSPE (Figure 5A). Additionally, western blot results confirmed the same protein expression pattern of p-gp in MCF-7R cells (Figure 5B). Furthermore, the mRNA of MDR1 was tested by RTPCR, and the MDR1 levels also decreased in P123-PEG2000-DSPE (Dox) and P123-PEG2000-DSPE compared with Dox, PEG2000-DSPE (Dox), and PEG2000-DSPE (Figure 5C).

Effect of P123-PEG2000-DSPE (Dox) on cell apoptosis

Apoptosis is the main way of cell death induced by cytotoxic drugs, and growing evidence shows that the changes in the apoptosis pathway is an important factor in MDR [20, 21]. The apoptosis of cells at 12 h after being treated with Dox, PEG2000-DSPE (Dox), P123-PEG2000-DSPE (Dox), PEG2000-DSPE and P123-PEG2000-DSPE was tested. Results showed that the apoptosis rate of BC cells in P123-PEG2000-DSPE (Dox) group was higher than that in both PEG2000-DSPE (Dox) and Dox groups. However, PEG2000-DSPE and P123-PEG2000-DSPE, the empty drug carriers, had no effects on apoptosis (Figure 6).

Discussion

MDR is a key factor in tumor treatment failure [22]. In this study, we constructed a micelle
mixed with Pluronic P123 and PEG2000-DSPE, and found that this mixed micelle had obvious advantages in inhibiting tumor cell resistance. Although Pluronic P85 is considered to have the strongest effect in reversing tumor MDR, its hydrophobic block is shorter, CMC value is larger, and solubilization ability is poor [23-25], so it is not considered as a good option for prescription. Pluronic P123 has good solubilizing ability and strong ability to reverse MDR [26]. Therefore, P123-PEG2000-DSPE constructed in this experiment could self-aggregate in aqueous solution to form blank micelles with PEG as hydrophilic segment and DSPE as hydrophobic segment. The hydrophobic core of P123-PEG2000-DSPE (Dox) was used to support Dox, while the hydrophilic outer shell maintained the stability of the drug-loaded micelles and reversed the MDR ability. After testing, the physicochemical properties of the P123-PEG2000-DSPE (Dox) met the requirements. The diameter of P123-PEG2000-DSPE (Dox) was 29±0.3 nm. The results showed that drug release from PEG2000-DSPE (Dox) or P123-PEG2000-DSPE (Dox) was downregulated at pH 7.4 (24% and 25%) relative to pH 5.0 (78% and 78%). It was similar of the release profiles of PEG2000-DSPE (Dox) and P123-PEG2000-DSPE (Dox) at

Figure 5. Effect of Dox, DSPE-PEG2000, P123-DSPE-PEG2000, DSPE-PEG2000 (Dox) and P123-DSPE-PEG2000 (Dox) on p-gp and MDR1 expression in MCF-7R cells. A: p-gp expression in MCF-7R cells measured by cell immunofluorescence; B: p-gp expression in MCF-7R cells measured by Western blot; C: MDR1 mRNA expression in MCF-7R cells measured by RT-PCR. **P<0.01 vs. P123-DSPE-PEG2000 (Dox).
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This result was consistent with a previous study [27]. Therefore, we investigated the reversal effects on BC MDR with P123-PEG2000-DSPE as a Dox carrier.

Among malignant tumors, the mortality rate of BC is second only to that of lung, gastric, hepatocellular, esophageal and colorectal carcinoma [28]. However, due to the existence of MDR, the curative effect of BC is still unsatisfactory [29]. It has been found that the drug efflux pump p-gp and MDR-related protein MDR1 are important to MDR; and MCF-7R is a p-gp- and MDR1-over-expressed BC drug-resistant cell line which can weaken apoptosis and reduce drug toxicity to cells via drug efflux [30]. Therefore, we chose MCF-7R cells as BC drug-resistant cells to investigate the role of Dox-loaded P123-PEG2000-DSPE on cytotoxicity, cellular endocytosis, cell resistance and apoptosis.

We found that the toxic effect of P123-PEG2000-DSPE (Dox) on MCF-7R cells was

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**Figure 6.** Effect of Dox, P123-DSPE-PEG2000, DSPE-PEG2000 (Dox) and P123-DSPE-PEG2000 (Dox) on MCF-7R apoptosis tested by the Annexin V-FITC/PI method.
enhanced by inhibiting the expression of resistance-related genes and promoting apoptosis. MDR of tumors is a key factor in the failure of tumor treatment. It was found that Pluronic P123 inhibited the expression of p-gp [31], and Pluronic P123/F127 mixed polymeric micelles also inhibited the expression of p-gp proteins in non-small cell lung cancer [32]. The present study also found that the expression of p-gp and MDR1 could be remarkably inhibited by the PEG2000-DSPE (Dox) or PEG2000-DSPE connected with Pluronic P123. Our findings suggest that Pluronic P123 is a key factor in inhibiting drug resistance in BC cells.

Apoptosis is the main pathway for cytotoxic drugs to induce cell death. There is increasing evidence that changes in the apoptotic pathways are important factors in tumor MDR. Pluronic P85 has the ability of regulating apoptosis signals. It can up-regulate BAX, PS3, APAF1, caspase3, caspase9 and other pro-apoptotic genes [33, 34], and Pluronic P123/F127 mixed micelles also promotes tumor cell apoptosis and reverses MDR [35]. Our study also found that Pluronic P123-PEG2000-DSPE (Dox) had a stronger promoting effect on the apoptosis of MCF-7R cells than PEG2000-DSPE (Dox) and Dox.

In summary, the present study first investigated the cytotoxicity and anti-MDR of P123-PEG2000-DSPE (Dox) systematically. From the present results, P123-PEG2000-DSPE (Dox), a well encapsulated mixed micelle which loaded Dox, could not only enhance tumor cellular uptake but also promote the toxicity of cells in vitro. Furthermore, overexpression of p-gp and MDR1 promotes tumor MDR and malignant progression [36-38]. Both P123-PEG2000-DSPE (Dox) and P123-PEG2000-DSPE-treated MCF-7R expressed much lower p-gp and MDR1 in the present study, indicating that P123-PEG2000-DSPE is a highly promising nano drug-loading system. In further research, it is needed to evaluate the MDR reversing potential of P123-PEG2000-DSPE-carriers in vivo.

Disclosure of conflict of interest

None.

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