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Anticancer Effects of Doxorubicin-Loaded Micelle on MCF-7 and MDA-MB-231, Breast Cancer Cell Lines

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ABSTRACT

Breast cancer is one of the most common cancers with high mortality, especially in women. Due to the severe side effects of chemotherapy drugs, scientists have tried to encapsulate these drugs in nanocarriers. The aim of this study was to analyze the anticancer effects of free doxorubicin and the doxorubicin-loaded OA400 micelle on MCF-7 and MDA-MB-231, breast cancer cell lines. The Drug loading (DL) and Encapsulation efficiency (EE) of DOX-loaded micelle were measured by spectrophotometry measurements. Dialysis bag method was used to evaluate the release of doxorubicin. We applied MTT assay in order to evaluate cell cytotoxicity of free and DOX-loaded micelle on MCF-7 and MDA-MB-231, breast cancer cell lines. The hydrophobic doxorubicin were successfully loaded into the OA400 micelles. The DL and EE was obtained 7.32 ±1.36% and 73.2 ±2.6%, respectively. The cumulative release rate of the drug was 27%, 22% and 12% at pHs of 4.5, 5.5 and 7.4, respectively. The IC_{50} value of the free DOX and DOX-loaded micelle in the MCF-7 cell line was 1.1 µg/ml and 1.8 µg/ml, respectively. Whereas the IC_{50} value for the free DOX and DOX-loaded micelle on MDA-MB-231 cell line was 1.38 µg/ml and 0.9 µg/ml, respectively. According to the results of this study, DOX-loaded micelle with less side effects had significant antitumor effects on breast cancer cell lines, especially MDA-MB-231 which could be as an alternative option for directed chemotherapy of breast cancer.

Key words: Breast Cancer, Doxorubicin**,** Micelle**,** Nanocarrier

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INTRODUCTION

Cancer is defined as the abnormal and uncontrolled growth of the cells affected by complex genetic and epigenetic DNA changes [1]. It is the second cause of mortality after cardiovascular diseases. Breast cancer is one of the most common types of cancer in women and the second most common cancer in both genders. According to the World Health Organization statistics in 2012, 1.67 million new cases have been reported with this type of cancer (25% of all cancers) [2-7].

Cancer treatment methods are chosen based on the type, location, stage of the disease and the status of cancer patients. These methods include surgery, radiotherapy, immunotherapy, hormone therapy and chemotherapy. Chemotherapy is one of the most important ways to treatment of cancer. However, today, so many chemotherapy drugs are used to treat various cancers that their use is limited because of some drawbacks such as serious side effects, low water solubility, and short circulation time [8-11].

Doxorubicin (DOX), chemically recognized as an anthracycline molecule, was isolated from the Streptomyces peucetius for the first time in the early 1950s and applied to the treatment of cancer

over the past 30 years [12]. It is one of the most commonly used drugs in the treatment of various cancers, such as breast, ovarian, stomach [13, 14]. DOX locates inside DNA, breaks the double-stranded DNA and reduces the synthesis of nucleic acid [15].

In order to overcome the specific constraints of traditional chemotherapy methods and to achieve more efficient therapies, anticancer drugs should be loaded into hydrophilic, biocompatible and nontoxic nano-carriers, with more durability in blood circulation. Also, these encapsulated drugs in nanoparticles can affect cancer cells with more tendencies and in a selective manner [16-22]. Doxil is the first drug loaded nanocarrier confirmed in 1995, in which liposomes modified by polyethylene glycol (PEG) was utilized to encapsulate DOX. By encapsulating DOX, the rate of heart damage decreased by one third [11]. Nano-carriers for drug transport systems are new therapeutic approaches designed for greater efficacy and eliminated side effects of the chemotherapy drugs. Several systems with various structures including liposomes, micelles, polymer-drug conjugates, dendrimers, silica nanoparticle, carbon nanotubes and metal nanoparticles have been introduced and used to treat various cancers [16-22].

The OA400 PEGylated micelle is defined as a polymeric nano-carrier derived from oleic acid. Stability, non-toxicity, neutrality of electrical load, biodegradability, easy preparation and low cost of production are the advantages of these nanoparticles in comparison with phospholipid carriers [23-25]. The high ability of OA400 micelle in gene delivery to cells has led to the emergence of the hypothesis that the OA400 micelle may deliver curcumin to cancer cells. It was also shown that the OA400 micelle alone were not toxic [26]. Other studies have reported the successful anti-cancer role of curcumin-loaded OA400 micelle [26, 27]. The aim of this study was to analyze the anti-cancer effects of free DOX and the DOX-loaded OA400 micelle on MCF-7 and MDA-MB-231, breast cancer cell lines.

MATERIALS AND METHODS

Doxorubicin hydrochloride (DOX-HCl) (Pharmacia, S.p.A, Milan, Italy) tetrahydrofuran (THF), triethylamine (TEA), diphenyl tetrazolium bromide (MTT), and oleyl chloride (Sigma-Aldrich, St. Lewis, USA) Gibco™ Roswell Park Memorial Institute (RPMI) 1640 Medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA and penicillin-streptomycin (Thermo Fisher Scientific). Ethanol, methanol, dimethyl sulfoxide (DMSO), chloroform and dialysis bags (Merck, Germany). All solvents were suitable for synthesis.

Synthesis of OA400 micelle

The OA400 micelle is defined as the PEGylated micelle derived from fatty acid units of oleic acid, designed and synthesized for the first time by Dr. Majid Sadeghizadeh's research group in Tarbiat Modares University, Iran. The OA400 micelle were synthesized by esterification reaction between oleyl chloride (0.3g) and polyethylene glycol 400 (4g) in the presence of triethylamine (1.2g) and chloroform as the solvent. Finally, the OA400 carrier was obtained after separating the triethylamine hydrochloride salt from the organic phase and evaporating chloroform at 40 °C under vacuum for 4 hours [26].

Preparation of DOX-loaded micelle (OA400)

DOX was loaded into micelles by dropwise addition of 10 mg OA400 micelle and 1 mg DOX in 2 ml DMSO. Then was added 10 ml deionized water under stirring at room temperature and was poured into a dialysis bag (MWCO, 12000 Dalton) and then was placed in 50 ml of water. The dialysis medium was changed four or four times. The contents inside the dialysis tube were lyophilized.

Cell lines and culture

The MCF-7 and MBA-MD-231 were purchased from the National Cell Bank at Pasteur Institute of Iran (Tehran, Iran). These cell lines were inoculated in DMEM and RPMI media, respectively. Cells were complemented with 100 mg/ml streptomycin, 100 U/ml penicillin and 10% v/v FBS. The cells were incubated at 37°C with 5% CO2 atmosphere.

Drug Loading (DL) and Encapsulation Efficiency (EE)

The DOX-loaded micelle lyophilized powder was dissolved in DMSO and was shaken vigorously for 5 minutes. Then, DL and EE of DOX-loaded micelle were measured three times in accordance with the standard curve by spectrophotometer (UV-160A Shimadzu, Japan) at the wavelength of 480 nm.

Stability

The stability of lyophilized samples of DOX-loaded micelle was assessed at 4°C for initial, 6 and 9 months. The particle size and polydispersity index

(PDI) were measured by Dynamic light scattering (DLS).

***Invitro* drug release**

Dialysis bag method was used to evaluate the release of doxorubicin from the nanocarier compound. One milliliter of the DOX-loaded micelle was poured into a dialysis bag (MWCO, 12000 Dalton) and then was placed in 50 ml of phosphate buffer saline (PBS) at a concentration of 0.1 M and various pHs of 4.5, 5.5 and 7.4 in a shaker incubator of 100 rpm at 37°C. Then, at time courses of 0, 1, 2, 4, 8, 12, 24, 48 and 72 hours, one milliliter of the samples was removed and replaced with one milliliter of fresh medium. The concentration of doxorubicin in each sample at the wavelength of 480 nm was measured three times in accordance with the standard curve by spectrophotometer (UV-160A Shimadzu, Japan).

Cytotoxicity assay

We applied MTT assay in order to evaluate cell cytotoxicity of free and DOX-loaded micelle on MCF-7 and MDA-MB-231, breast cancer cell lines. The appropriate cell number (5000 cells per well) was transported to the 96-well plate with a final volume of 200µl of the culture medium. After 24 hours, when the cells adhered to the bottom of the plate, we poured different concentrations of free and DOX-loaded micelle (0-50 µM) into each well and pipetted it well. Each concentration was tested as a triple repeat. After 24, 48 and 72 hours, 20 µl of MTT solution (5mg/ml) was added to each well and mixed thoroughly until well dissolved. MTT reagent, a yellow tetrazolium salt, is absorbed into the mitochondria of the active cells metabolically and due to the activity of dehydrogenase enzymes, purple formazon crystal is produced . Approximately 4 hours later, when purple crystals were formed, the superficial solution was completely removed and 200 µl DMSO was added. Then, after 30 minutes, its absorption at the wavelength of 480 nm was read by the Microplate ELISA Reader (Awareness Technologies Stat Fax 2100). The absorption ratio in the treatment group cells to the control group (zero concentration of drug) would indicate the survival of the cells in each concentration. The concentration in which 50% of the cells were killed by the drug was considered as lethal concentration (LD_{50}: Lethal Dose). Data from 3 separate tests were presented as mean ± SD.

RESULTS

Different concentrations of hydrophobic doxorubicin (0.1-2mg/m2) were successfully loaded into the OA400 micelles. The ratio of 1 to 10 is the highest DL and EE was obtained 7.32 ±1.36% and 73.2 ±2.6%, respectively, selected for further analyses. The stability of the DOX-loaed micelle was analyzed by measuring the size and PDI at 4°C for 6 and 9 months using DLS analysis. The results of this study showed no significant change in the size and PDI at these times (Table 1).

To evaluate the DOX release from the nanocarrier compound, dialysis bags were used at different pHs of 4.5, 5.3 and 7.4 at time courses of 0, 1, 2, 4, 8, 12, 24, 48 and 72 hours. DOX release had a two-phase pattern: the first phase included an explosive release at the start (first 8 hours) and the second phase included a slow but continuous release for a long time. The cumulative release rate of the drug was 27%, 22% and 12% at pHs of 4.5, 5.5 and 7.4, respectively (Figure 1). The release rate of the drug was higher at PH of 4.5 than other PHs. Accordingly, the OA400 nanocarrier was good for trapping doxorubicin in physiological pH (7.4) and release in acidic pHs (4.5 and 5.5). From the targeting tumors viewpoint, an increase in the release in low pHs seems to be an advantage.

The cell toxicity of free DOX and DOX-loaded micelle and empty micelle on MCF-7 and MDA-MB-231, breast cell lines in three time intervals of 24, 48 and 72 hours were determined by MTT assay. The results of this study showed the free DOX and DOX-loaded micelle causes a decrease in the survival of the MCF-7 and MDA-MB-231, breast cell lines depending on dosage and time. The results suggest that the best effect of the free DOX and DOX-loaded micelle on MCF-7 and MDA-MB-231, breast cell lines were observed in the 48 hours after the treatment. The effect of free DOX on the MCF-7 cell line was greater than DOX-loaded micelle, whereas the effect of DOX-loaded micelle greater than free DOX on the MDA-MB-231 breast cell line. The IC_{50} value of the free DOX and DOX-loaded micelle in the MCF-7 cell line was 1.1 µg/ml and 1.8 µg/ml, respectively (Figure 2). Whereas the IC_{50} value for the free DOX and DOX-loaded micelle on MDA-MB-231 cell line was 1.38 µg/ml and 0.9 µg/ml, respectively (Figure 3). According to the results of MTT assay, we didn't observe any significant toxicity for empty micelle on the mentioned two cell lines in above.

Table 1. The stability of DOX-loaded micelle in 4°C after 6 and 9 months by DLS analysis

Formulation	Test	Initial Mean±SD	6 months Mean±SD	9 months Mean±SD
DOX-loaded micelle	Size	69.56±27.43	68.78±33.19	68.43±28.81
	PDI	0.30	0.27	0.27

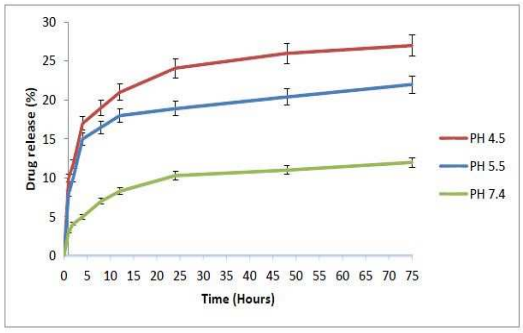

Figure 1. In vitro drug release of DOX-loaded micelle (OA400) at three different pH levels.

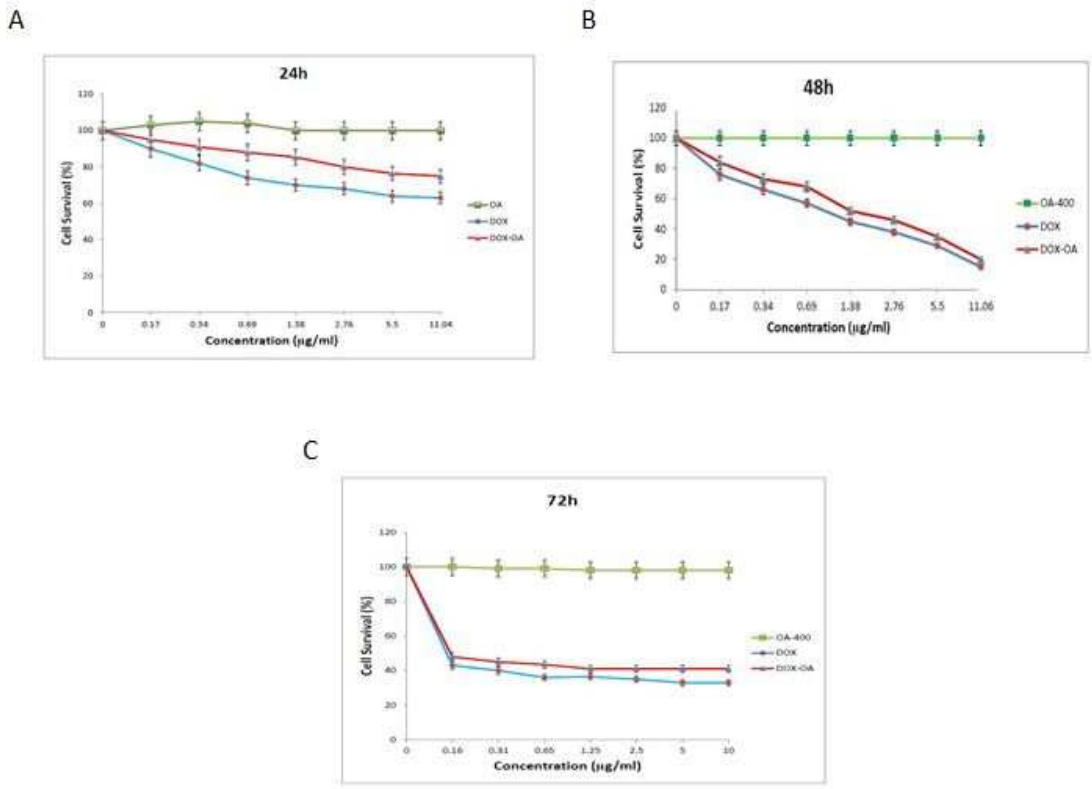

Figure 2. Cytotoxicity assay of OA-400 empty, DOX, and DOX-loaded micelle on MCF-7 cell line after (A) 24 h, (B) 48 h, (C) 72 h.

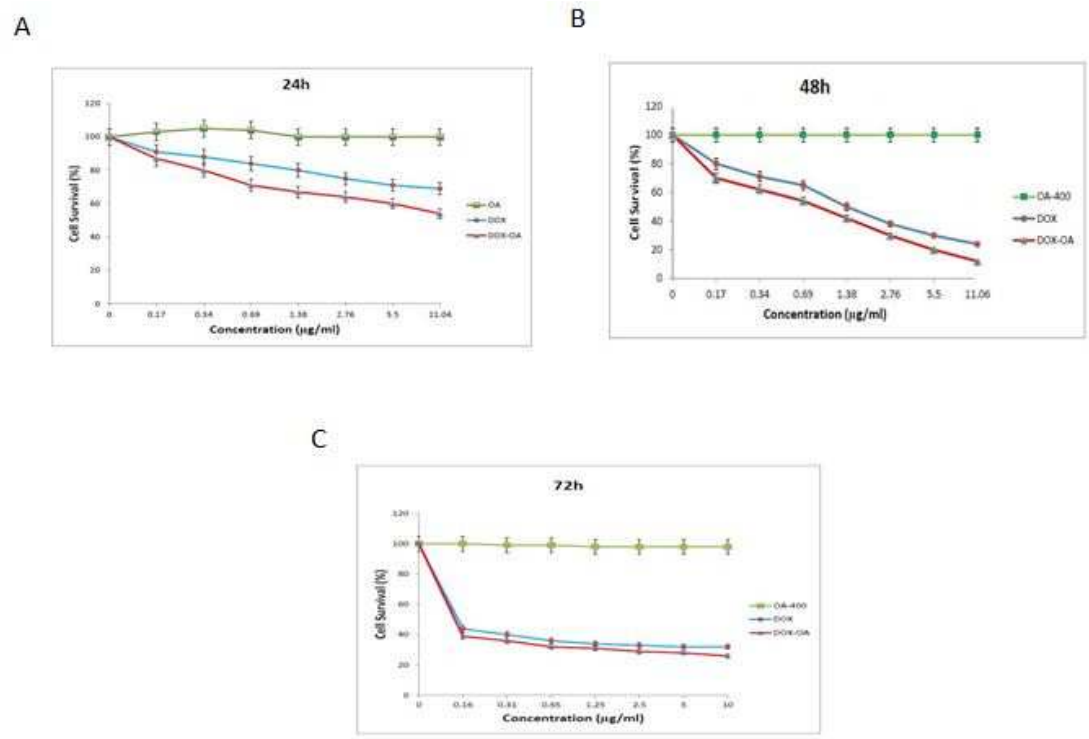

Figure 2. Cytotoxicity assay of OA-400 empty, DOX, and DOX-loaded micelle on MDA-MB-231 cell line after (A) 24 h, (B) 48 h, (C) 72 h

DISCUSSION

Breast cancer is one of the most common cancers with high mortality, especially in women [13]. Due to the severe side effects of chemotherapy drugs, scientists have tried to improve the anti-cancer effects of these drugs by using appropriate nanocarriers while eliminating the side effects of drugs. In recently years, some of the most important chemotherapy drugs have been encapsulated in appropriate nanostructure and anticancer effects of these compounds have also been investigated [16-23]. In the present study, after the encapsulation of the doxorubicin in OA400 PEGylated micelle, we studied the effects of this nanoparticle on MCF-7 and MDA-MB-231, breast cancer cell lines.

The results revealed that DL and EE of this nanoparticle were 7.32 ±1.36% and 73.2 ±2.6%, respectively, indicating successful loading of doxorubicin in OA400 micelle. In addition, the results of physical stability of the nanoparticle showed that the size and drug content were stable after 6 and 9 months. The invitro drug release of this nanoparticle was assessed using dialysis bag method at three different pH levels (4.5, 5.5 and 7.4). The release pattern had two phases. The first phase included an explosive and rapid release followed by the second phase that is slow but steady. The release rate in acidic pHs of 4.5 and 5.5 was higher than pH 7.4, which seems to be an advantage due to the acidity of the pH inside the tumor.

MTT assay was used to evaluate the anticancer effects of free DOX and DOX-loaded micelle on MCF-7 and MDA-MB-231, breast cancer cell lines. The results showed that the OA400 micelle alone had no toxic effects on the cell lines. Moreover, the toxic effects of these compounds on the cell lines were dependent on the time and dosage of the used drug. Furthermore, the effect of free DOX on the MCF-7 cell line was greater than DOX-loaded micelle, which could be due to the easier release of the drug into the cell, access to the nucleus and its effect on DNA. However, on the other hand, DOX-loaded micelle may have fewer side effects for vital tissues, especially the heart, along with antitumor effects. However, in the MDA-MB-231 cell line, DOX-loaded micelle had more antitumor effects compared to the free DOX, which could be due to higher levels of release and more permeability of this cell line.

In general, according to the results of this study, DOX-loaded micelle with less side effects had significant antitumor effects on breast cancer cell

lines, especially MDA-MB-231, cell lines, which could be further analyzed as an alternative option for directed chemotherapy of breast cancer.

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