RESEARCH ARTICLE

Cytotoxicity Enhancement of Paclitaxel by Loading on Stearate-g-dextran Micelles on Breast Cancer Cell Line MCF-7

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Abstract

   Objective: Paclitaxel (PTX) is a chemotherapeutic agent used for treating breast cancer. The study aimed to prepare PTX loaded dextran stearate (Dex-SA) and evaluate its efficacy against human breast cancer cell line MCF-7. **Methods:** Dex-SA/PTX micelles were prepared by dialysis method. The micelles size, zeta potential and particle size distribution were measured by dynamic laser light scattering method. Amount of loaded PTX on the polymer measured by HPLC. Release profiles of the drug from the micelles were obtained in buffer (phosphate pH=7.4). Then the cytotoxicity of blank micelles, Dex-SA/PTX micelles and free PTX were evaluated in the MCF-7 cells by MTT method. **Result:** Loading efficiency of PTX on the Dex-SA was measured about 84.24±9.07%. The smallest particles size was about 193.9±7.1 nm but the other formulation with larger particle size had better zeta potential (-33.5±6.74 mV). The drug release from the micelles was slowly and reached steady state after about 12 hours. The cytotoxicity experiment showed that Dex-SA/PTX micelles have more cytotoxicity compared to free PTX against MCF7 cell lines. **Conclusions:** Dex-SA polymeric micelle is a suitable carrier for hydrophobic cytotoxic drugs such as PTX.

   Keywords: Paclitaxel- Dextran Stearate- MCF-7- Polymeric Micelles

Introduction

   Breast cancer is one of the most lethal gynecologic malignancies and the second leading cause of cancer death among worldwide in women (Vaidya et al., 2010). According to available statistics, breast cancer has been growing promptly in developing countries (Mariotto et al., 2011; Warner, 2011), such as Iran (Mousavi et al., 2009). Chemotherapy is a key treatment option for breast cancer, and its effects dramatically depended on the efficiency of drug delivery system (Ebrahimifar et al., 2017).

   Paclitaxel (PTX) is belonging to taxane family of chemotropic drugs. Main applications of the drug are to treat ovarian, breast, lung and pancreatic cancers (Varshosaz, 2012). PTX enhances the polymerization of tubulin to stable microtubules and stabilizing them against depolymerization (Du et al., 2010). Unfortunately, this drug has a short half-life, so in order to achieve the required concentration during appropriate periods, it is necessary to use frequently high doses of the drug. This leads to inadequate effect for treatment and several adverse effects. Other items that create restrictions on treatment with this drug are weak solubility in aqueous solutions

and lack of stability of the drug (Ta-Chung et al., 2005; Weaver, 2014).

   In most cases, ingratiation of drugs into nanoparticle carriers may change the properties of these drugs. Some of these desirable changes include increased cellular uptake of them (Ebrahimifar et al., 2017), protection of drugs from metabolism, elimination from the body and also help to solubilize insoluble drugs (Zhou et al., 2013).

   Dextran is a member of natural branched polysaccharides that has been studying as polymeric carriers in novel drug delivery systems (Varshosaz, 2012). Amphiphilic derivatives of dextran can be produced by conjugation of it with lipophilic chemical compounds (Nagahama et al., 2015). Stearate-g-dextran (Dex-SA) was synthesized via an esterification reaction between the hydroxyl group of dextran and carboxyl group of stearic acid (Du et al., 2010). This reaction leads to the production of surface active amphiphilic molecules that can be formed as core-shell nanostructures or polymeric micelles within an aqueous media spontaneously (Varshosaz et al., 2012). These micelles may be useful for drug delivery, especially anti-cancer drugs. For instance, Du, Weng et al. showed that Dex-SA micelles had excellent cell internalization

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ability, which could deliver doxorubicin into tumor cells and could prolong in vitro drug release (Du et al., 2010).

In this study, to improve the PTX efficacy, Dex-SA was used as a drug carrier. Nanoparticles were characterized in terms of zeta potential, size, morphology and drug retention capability. Finally, the efficacy of PTX loaded Dex-SA (Dex-SA/PTX) micelles was evaluated on the breast cancer MCF7 cell lines.

Materials and Methods

All chemicals were obtained from Merck, Darmstadt, Germany unless otherwise stated. Dextran, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), stearoyl chloride and dialysis tubs were purchased from Sigma (Poole, England). All solvents and lithium chloride were purchased from Samchon (Korea). PTX was prepared from Sobhan oncology pharmaceutical company (Rasht, Iran). It should be noted that all solvents were of HPLC grade.

Synthesis of dextran stearate

Dex-SA was synthesized as described in our previous studies (Varshosaz et al., 2012; Varshosaz et al., 2014). Briefly, 2 g lithium chloride and 1 g dextran were dissolved in 20 mL of dimethylformamide (DMF) and stirred at 120 °C for 2 hours under nitrogen atmosphere. It cools down to 80 °C then 180 mg (1.48 mmol) of dimethyl aminopyridine (DMAP) was added. 420 μL (1.25 mmol) of stearoyl chloride dissolved in 20 mL of DMF and drop wised was added to the last solution in 1 hour and stirred for overnight. The reacting mixture was cooled and precipitated with 200 mL of methanol. The yellow precipitate was filtrated, washed and dried in vacuum oven.

Preparation of Dex-SA/PTX micelles

PTX was loaded into the Dex-SA p olymeric micelles via membrane dialysis method (Attia et al., 2013). Briefly, 10 mg (in F10 formula) and 20 mg (in F20 formula) of PTX and 100 mg of Dex-SA were dissolved in 7 mL dimethyl sulfoxide (DMSO) and dialyzed in 1 L distilled water for 2 hours then water was changed and dialyzed was continued overnight at room temperature. Finally, the drug-loaded micelles were lyophilized and kept 4 °C for further evaluations. To measure the drug loading (DL) capacity, 4 mg of the lyophilized powder was dissolved in 1 mL DMSO and 1 mL of this solution was diluted to 10 mL by acetonitrile. The solution was centrifuged at 2000 rpm and PTX concentration was analyzed using HPLC instrument equipped to UV detector at 227 nm. The encapsulation efficiency (EE) and DL were calculated by the following equations:

$$EE\ (\%) = \frac{PP - PS}{PP} * 100 \qquad (1)$$

$$DL\ (\%) = \frac{C}{W} * 100 \qquad (2)$$

In formula 1, EE, Encapsulation efficiency; PP, primary PTX; PS, PTX in the supernatant. In formula 2, DL: Drug loading, C: PTX content in the micelles and W: weight of micelles.

Drug release studies

The dispersion of 5 mg different lyophilized powders was prepared in 2 mL distilled water. The resulting liquids were placed in a dialysis bag, and the bag was immersed in the bottle with 100 mL of phosphate buffer solution (PBS) with pH=7.4 and 0.1% tween 60. The release mediums were agitated by 200 rpm for 3 days at 37 °C. One mL samples were withdrawn at 0, 2, 5, 12, 24, 48, 72 h intervals and replaced with fresh PBS maintained at the same pH and temperature.

Apparatus and procedure for HPLC analysis

The content of PTX in the samples was determined by the HPLC method. a system equipped with a LC-20AD pump, a SPD-20A UV detector (Shimadzu, Kyoto, Japan) and operated at 214 nm. A reversed-phase column (prefectSil target 5 μm C18, 250 mm × 4.6 mm, MZ, Germany) was used at room temperature. The mobile phase consisting of acetonitrile, and water (70:30, v/v) was freshly prepared for each run and degassed before use. The mobile phase was delivered at a flow rate of 1.0 mL/min.

Characterization of Dex-SA/PTX micelles

Size, zeta potential, and polydispersity index (PDI) of micelles were measured by dynamic laser light scattering method (Quader et al., 2014). Five mg of different lyophilized samples were dissolved in 5 mL water and analyzed at 25°C. The particles size, poly disparity index and zeta potential were measured in triplicate. In addition, the scanning electron microscope (SEM) analysis was done to describe the morphology by means (KYKY-EM3200) at an operating voltage of 26 kV.

MCF-7 cell line culture

The MCF-7 cell line was obtained from Pasture Institute, Tehran, Iran. The cells were grown in 25 cm² flasks in RPMI-1640 supplemented with 100 μg/mL streptomycin, 100 μg/mL penicillin and 10% (w/v) heat-inactivated fetal bovine serum albumin. The cells were maintained in an atmosphere of humidified 5% CO_2 at 37 °C. The culture medium was renewed every other day.

Analysis of cell viability

The cell viability assay was carried out according to the protocol described by Yang et al., (2014). Preliminary experiments were conducted to standardize the number of cells to be seeded onto the 96 well plates. 10×104 cells were seeded to each well. Cells were allowed to attach (for 24 hours), and then treated with different concentrations of free PTX and Dex-SA/PTX micelles. The medium containing the active fraction was removed after 48 hours incubation, washed with PBS buffer and 100 μL of the MTT solution (5mg/mL) was added to each of the 96 wells. After 4 hours of incubation at 37 °C, the solution was removed and 100 μL of DMSO was added to each well to solve insoluble formazan precipitate. The

absorbance of the wells was read on by the plate reader (Tecan, Austria) at 570 nm.

Statistical analysis

All the results are presented as mean ± SD. Statistical analysis of the data was performed by comparing the means through one-way ANOVA. All statistical analyses were performed using the SPSS and P values ≤ 0.05 were considered as statistically significant.

Results

Physicochemical properties of Dex-SA/PTX micelles
As shown in Table 1, although there is no significant difference in the EE between F10 and F20 formulations, a significant difference was observed in size ($P<0.001$), zeta potential ($P<0.001$), DL ($P<0.01$) and PDI ($P<0.001$) levels. In addition, SEM findings showed that the micelles were quite spherical, and the SEM image was confirmed the particles size (Figure 1).

Drug release studies

The release profiles of both Dex-SA/PTX micelles were approximately similar. Both formulas had a burst release in first 2 hours. Thereafter the drug was released by nearly zero order kinetic. The release pattern of the F10 formula was more uniform than the F20 profile. As can be seen in the profiles F20 formula was reached nearly to steady state after 60 minutes (Figure 2).

Cytotoxicity assay

As shown in Figure 3, the cell proliferation studies
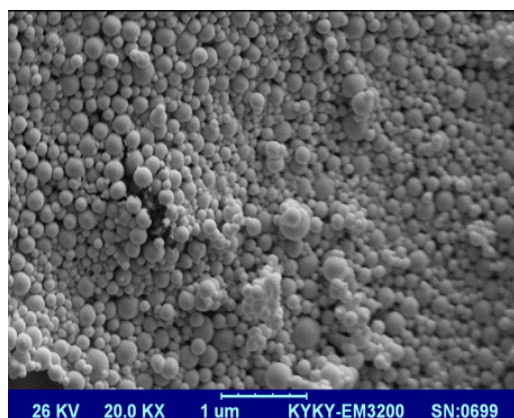

Figure 1. SEM Image of Dex-SA/PTX Micelles with Scale bar=1 μm.

Table 1. Physicochemical Properties of Dex-SA/PTX micelles

Formula	F10	F20
EE %	84.9±14.7	74.3±9.0
DL %	7.7±1.3	12.3±1.5**
Particle size (nm)	193.9±7.1	468.9±16.1***
Zeta-potential (mV)	-20.8±5.0	-33.5±6.7***
PDI	0.241±0.019	0.518±.061***

EE, entrapment efficiency; DL, drug loading; PDI, Polydispersity index; Data are expressed as mean ± SD from three independent experiments.
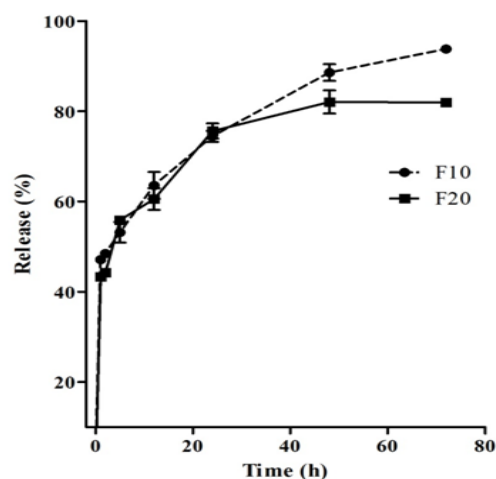

Figure 2. Release Profile of PTX Release from Dex-SA/PTX Micelles. Data are expressed as mean ± SD (Standard Deviation) from three independent experiments.
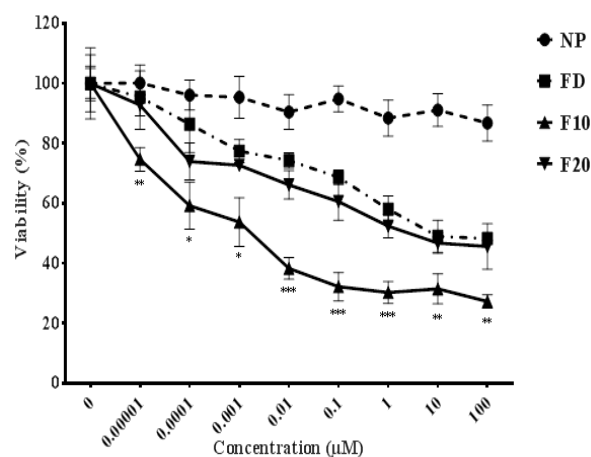

Figure 3. Cytotoxic Effects of Free PTX and Dex-SA/PTX Micelles on MCF7 Cell Lines. Data are expressed as mean±SD from three independent experiments. Significantly different from F10 formulation: * (P < 0.05), ** (P < 0.01), *** (P < 0.001).

indicate that there is no significant difference between control and pure nano-carriers groups, in the range of doses 0.00001-100 μM. In addition, a significant decrease was observed on MCF-7 cell proliferation, in response to different concentrations of free PTX (in the range of 0.001-100 μM) after 48h in comparison with control. This range of dose was determined for F10 and F20 formulations 0.00001-100 μM and 0.0001-100 μM respectively. Among these, F10 efficacy was significantly more than F20 formulation, in the range of 0.00001-100 μM, when compared with together ($p<0.001$).

Discussion

PTX appears to be one of the most promising drugs used in treatment of various cancers. However, PTX has very poor aqueous solubility, which significantly limits its bioavailability and therapeutic efficacy (Zhou et al.,

2013). In order to increasing the solubility of commercial PTX formulation that currently is available in the market, Cremophor EL (CrEL) was used as a solubility enhancer. This surfactant is not inert pharmacologically and biologically. It was involved with toxic side effects, such as neuropathy and hypersensitivity reaction (Ta-Chung et al., 2005). Therefore, replacing the surfactant is considered to reduce such complications.

In the present study, Dex-SA polymeric micelles were used as a carrier for increasing solubility and efficacy of PTX. The results indicated that free PTX and Dex-SA/PTX micelles were significantly effective on inhibition of the growth of MCF-7 cell line in dose- and time-dependent manner. Polymeric micelles are the suitable carrier for water-insoluble drugs such as PTX. Micelles are capable of encapsulating significant amounts of drugs at their hydrophobic cores (Ahmad et al., 2014). The hydrophilic shells and their small size guarantees long circulation of micelles as well as the cargo. Moreover, they have the ability of exclusive extravasation from blood vessels into the tumors tissues despite this extravasation from vessels in healthy tissue less frequently outcropped (Cabral et al., 2011).

The Table 1 exhibit efficient drug encapsulation in both formulation of Dex-SA/PTX micelles. There is no significant difference in the EE of the micelles. In comparison with other drugs that are encapsulated in the polymer (Dex-SA), EE of PTX has been better than doxorubicin hydrochloride and lower than Etoposide (Varshosaz et al., 2012). Although PTX content of the F20 was more than the F10 formulation, the F10 particle size is more suitable for intravenous administration and accommodation in solid tumors. According to the findings of previous studies which is done in xenograft tumor models, have been shown that smaller size particles had better penetration and retention in solid tumors (Patlolla and Vobalaboina, 2005; Reddy and Venkateswarlu, 2005; Meng et al., 2011).

Based on the results of PDI in Table 1, the formula F10 had narrower particle size distribution indicated more homogeneous size distribution of the particles. These significant differences between the particles size distribution of drug-loaded formulations may be due to the difference in the amount of drug-loaded into the particles. The SEM results also confirmed the preparation of spherical micelles with the smooth surface. Zeta potential of the F10 formulation was significantly less negative than the F20 formula. Since this surfactant (Dex-SA) is a nonionic surface active agent, the surface charge of the nanoparticles which they were made is determined mostly by their drug content (Varshosaz et al., 2012). The F10 micelles had a lower drug to polymer ratio, so the charge of these particles is lower than the other formula.

The use of polymeric micelles as drug delivery platforms could play an important role in slowing the release of the drug (Yuan et al., 2014). Although the F20 size and PDI were more than F10 formulations, therefore it is expected that the rate of drug release is more uneven and slower in the formulation F20.

Slow release pattern of PTX in the two formulas within 72 hours were approximately equal, and up to 80% of the drug that loaded in both formulas have been released at the end of the period. Our findings showed that drug release from both formulas was slow and have been observed a burst release in first 2 hours (Figure 2). Burst release in the first 2 hours of study may be indicated the release of the adsorbed drug on the surface of the micelles. This initial Burst release has been observed less or more (between 40 and 60 drugs loaded) in previous studies that have been conducted on drug release from other polymeric mixtures (Dahmani et al., 2012; Zhang et al., 2012; Zhao et al., 2012). Thereafter the drug was released by nearly zero order kinetic that could be explained by deposition of the drug into hydrophobic core of polymeric micelles due to hydrophobic interactions between fatty acid residues and the hydrophobic drug and diffusion of the drug through dextran hydrophilic membrane, Which surrounds the hydrophobic core in water (Varshosaz et al., 2012; Wang et al., 2017). To achieve lower release rates and burst effect, in some of the cases (chitosan grafted stearic acid) hydrophilic block of polymeric surfactant has been crosslinked after PTX loading by glutaraldehyde (Hu et al., 2006).

Concerning the cytotoxicity, our data showed that blank Dex-SA polymeric micelles had no significant effect on MCF-7 cell proliferation and its safety range was determined 0.00001- 100 µM. In the previous studies, similar results about the Dex-SA carrier has been observed in CT-26 (Varshosaz et al., 2012), MCF-7 and A549 cell lines.

As shown in Figure 3, it was found that Dex-SA/PTX micelles have more cytotoxicity compared to free PTX against MCF7 cell lines. In addition, we found that the cell toxicity of F10 was significantly higher than F20 in MCF7 cell lines. Since the cytotoxicity of PTX is directly related to the amount of drug that enters the cell (Liu et al., 2015), this phenomenon may be related to further penetration of the F10, due to the smaller size, into MCF7 cell lines.

The results of the study clearly indicate that Dex-SA polymer can play the role of a good carrier for PTX delivery to cancer cells. The particle size of the micelles was about 200 nm that it seems to have a good permeability in solid tumors. There is optimal ratio for PTX to Dex-SA, which can cause the formation of micelles, with desirable physicochemical properties for drug delivery. The ratio is close to formula F10 which has the ability to release PTX slowly for about 72 hours. Moreover, cytotoxicity evaluation demonstrated that Dex-SA/PTX micelles have more potency than free PTX on MCF7 cell line. This phenomenon may be due to Dex-SA ability to increase cellular uptake of drugs by phagocytosis, which can improve the efficiency of therapeutic drug and reduce its adverse effects. However, further studies are needed to investigate anti-tumor activity of Dex-SA/PTX micelles and its distribution in xenografts models.

Statement conflict of Interest
The authors declare that there is no conflict of interest.

Acknowledgments

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