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PRECLINICAL STUDIES

Antitumor activity of raloxifene-targeted poly(styrene maleic acid)-poly (amide-ether-ester-imide) co-polymeric nanomicelles loaded with docetaxel in breast cancer-bearing mice

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Summary *Purpose* Raloxifene (RA) receptors have over-expressed GPER-positive breast cancer tumors. The purpose of this work was to evaluate the antitumor activity and pharmacokinetic behavior of docetaxel (DTX), loaded in RA-targeted nanomicelles, which were designed to overcome a lack of specific distribution and inadequate DTX concentration in tumor tissues, as well as its cytotoxicity and damage to normal tissues. *Methods* DTX-loaded RA-targeted poly(styrene maleic acid) (SMA)- poly(amide-ether-esterimide)-poly(ethylene glycol) (PAEEI-PEG) nanomicelles were prepared; then, their antitumor activity and survival rate were studied in MC4-L2 tumors induced in BALB/c mice. The pharmacokinetics of DTX-loaded SMA-PAEEI-PEG-RA micelles was also investigated in comparison with free DTX. *Results* DTX-loaded SMA-PAEEI-PEG-RA micelles inhibited tumor growth considerably and increased animal survival as compared to free DTX and non-targeted micelles. SMA-PAEEIPEG-RA micelles enhanced significantly the area under the curve (AUC0-∞) 1.3 times as compared to free DTX and reduced clearance (CL) from 410.43 ml/kg.h (for free DTX) to 308.8 ml/kg.h (for SMA-PAEEI-PEG-RA micelles). Volume of distribution (Vdss) was also reduced 1.4 times as compared to free DTX. RA-targeted micelles increased tumor inhibition rate (TIR) 1.3 times and median survival time (MST) >1.5 times compared to free DTX. Percentage increase in life span (%ILS) was also enhanced significantly from 41.66% to >83.33% in MC4-L2 tumor-bearing BALB/c mice. *Discussion* All studies in this work showed the potential of DTX-loaded SMA-PAEEI-PEG-RA micelles in the treatment of GPER-positive receptor breast cancer tumors.

Keywords Poly(styrene maleic acid) · poly(amide-ether-ester-imide)-poly(ethylene glycol) · targeted micelles · raloxifene · docetaxel

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Introduction

Cancer is one of the main causes of morbidity and mortality among people around the world caused by an uncontrolled growth of cells [1, 2]. According to the American Cancer Society, breast cancer is one of the four most common types of cancer in the United States [3]. Current strategies for breast cancer treatment consist of chemotherapy, and surgery and radiation (alone or together) [4]. Taxanes (paclitaxel and docetaxel) are the most current chemotherapeutic agents used for the treatment of breast cancer [5]. Docetaxel (DTX), as a second generation of taxanes, is a poor water-soluble agent with a low bioavailability similar to other chemotherapeutic drugs; so, it is formulated with toxic solvents [6, 7]. Moreover, the low molecular weight of DTX causes a high volume of

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distribution, lack of specificity, and easy clearance from the body. All the aforementioned reasons contribute to an insufficient concentration of this chemotherapeutic drug in tumor tissues, as well as to its cytotoxicity and damage to normal tissues [8]. Nanotechnology solves the problems jointly with traditional chemotherapy because of the unique nanoscale size of the drug carrier. Nanocarriers (especially polymeric nanoparticles), as drug reservoirs, can be used for the formulation and encapsulation of chemotherapeutic agents. Polymeric micelles can increase the therapeutic performance of the drug due to specific delivery to tumor site; they can also improve bio-distribution and diminish its cytotoxicity in normal tissues [1–3]. They consist of a hydrophilic shell and a hydrophobic core that can solubilize drugs with low water solubility. Hydrophilic polymers like polyethylene glycol (PEG), as shell forming polymers that are adsorbed or covalently attached to the core segment of the micelles, prevent micelle uptake by macrophages and prolong efficient plasma circulation time of the loaded drug [9–11]. Owing to the hydrophilic nature of PEG, it can form a brush-like shell in an aqueous environment and its steric repulsion effects substantially reduce nonspecific interactions of nanomicelles with proteins (opsonization) and complement activation [11].

Tumor targeting consists of both active and passive targeting. In passive targeting, nanocarrier drug delivery systems can be retained and accumulated in the tumor environment by extravasation across fenestrated and leaky tumor capillaries. This uptake process is known as the enhanced permeability and retention (EPR) effect [12–14]. The accumulation of passive nanomedicines in tumor tissues depends on surface properties, the particle sizes of nanoparticles, and the vascular network of the tumor tissue [1]. An active targeted delivery system means that targeting ligands (like antibodies, peptides, sugars, nucleic acids, and polymers) are connected to the surface of nanocarriers and the ligands are recognized by overexpressed receptors present in targeted tumor cells [3, 13]. The entrance of cytotoxic drugs into cancer cells is done by the interaction of targeting molecules with their receptors and via endocytosis-dependent mechanisms that lead to high intracellular drug concentrations [1].

The G-protein-coupled estrogen receptor (GPER), also called GPR30, is one of the members of the G-protein-coupled receptor (GPCR) superfamily with approximately 800 known members; it is characterized by seven transmembrane helices and is most expressed at cell surfaces [15, 16]. GPR30 is a membrane estrogen receptor (mER) that has a single binding site with high affinity and limited capacity specifically for estrogens. This peptide receptor is widely distributed in different cell types like neural, ovarian, placental, prostate, heart, vascular epithelium, hepatic, and lymphoid tissues [17].

GPR30 may also play an important role in cancer, especially in hormone-sensitive tumors. This receptor is associated with the initiation, progression, and/or poor prognosis of breast cancer, endometrial carcinoma, and ovarian cancer. In patients with high expression of GPR30, survival is significantly decreased [16, 18]. The expression of GPR30 is high in estrogen-positive receptor human breast carcinoma cell lines (MCF7, T-47D, and MDA-MB-361) but its expression is low in estrogen-negative receptor human breast carcinoma cell lines (BT-20, HBL-100, and MDA-MB-231) [19]. There are many natural and synthetic estrogenic and anti-estrogen compounds (including phytoestrogens, mycoestrogens, and xenoestrogens) that are examples of ligands for GPR30 [18].

Raloxifene (RA) is a synthetic compound and a ligand for GPR30 receptors. RA, as a selective estrogen-receptor modulator (SERM), is an estrogen agonist in the cardiovascular and skeletal system but an estrogen antagonist in the uterus and breast tissues. It reduces the incidence of invasiveness of estrogen-positive receptor breast cancer as compared to the placebo [18, 20, 21].

In our previous study, we used poly(styrene maleic acid) or SMA and a synthetic PEG derivative named poly(amide ether ester imide)-poly(ethylene glycol) or PAEEI-PEG for the preparation of a novel polymeric nanomicelle, targeted by RA because of a high expression of GPR30 receptors in estrogen-positive breast cancer cells for the first time, loaded with DTX [23]. In the present work, the anti-tumor efficacy and pharmacokinetic behavior of this targeted nanocarrier is studied in breast cancer-bearing BALB/c mice.

Materials and methods

Materials

RA was gifted by the Iran Hormone Company. DTX was purchased from Cipla (India). SMA-PAEEI-PEG-PEG polymeric micelles, loaded with DTX, were prepared in our research center at the School of Pharmacy (Isfahan University of Medical Science). Tween 80, ethanol, dicyclohexyl carbodiimide (DCC), dimethyl amino pyridine (DMAP), dimethyl sulfoxide (DMSO), N-methylpyrrolidone (NMP), diethyl ether (analytical grade), acetonitrile (ACN), and HPLC-grade water were purchased from Merck Company (Germany). The dialysis membrane (6–8 KDa) was purchased from Sigma Aldrich (St Louis, USA). Diazepam was provided by Farabi Company (Iran). Fetal bovine serum (FBS), trypsin, phosphate buffer saline (PBS), penicillin, streptomycin, and Dulbecco's Modified Eagle Medium (DMEM F12) were provided by Gibco Laboratories (USA). The MC4-L2 murine breast cancer cell line was purchased from the Iranian Institute of Biological and Genetic Resources.

Preparation of DTX-loaded RA-grafted SMA-PAEEI-PEG and non-targeted SMA-PAEEI-PEG micelles

SMA-PAEEI-PEG-RA and SMA-PAEEI-PEG co-polymers were synthesized as previously reported [22, 23]. The dialysis method was used for preparing the DTX-loaded micelles. Briefly, 5 mg DTX and 10 mg SMA-PAEEI-PEG-RA or SMA-PAEEI-PEG were dissolved in NMP, transferred to dialysis bag (6–8 KDa), immersed in water, and dialyzed at 37 °C. Thereafter, the suspension inside the bag was freeze-dried and studied for particle size, polydispersity index, (PDI), and zeta potential analysis by Malvern Zetasizer (ZEN3600, UK).

Determination of critical micelle concentration (CMC)

Critical micelle concentration (CMC) was measured by a spectrofluorimeter (LS-3, Perkin Elmer, USA) using pyrene as a fluorescent probe. Ascending aqueous solutions of co-polymers with concentrations ranging from 2.5 μg/ml to 100 μg/ml were prepared in glass tubes and mixed with an appropriate amount of pyrene stock solution (6×10^{-6} M in acetone) to get the final pyrene concentration of 6×10^{-7} M. The mixtures were then allowed to shake for 24 h at 37 °C in darkness until the acetone evaporated completely. Pyrene excitation at a fixed-emission wavelength of 390 nm was scanned between 280 and 480 nm by the spectrofluorimeter. The maximum fluorescence intensity of pyrene in water was $\lambda_{exc} = 303$ nm and for pyrene in the presence of micelles was $\lambda_{exc} = 335$ nm. The plot of the intensity of I_{335}/I_{303} ratio of the pyrene against the logarithm of the polymer concentration was used to calculate the CMC values.

Determination of drug encapsulation efficiency (EE)

Reverse-phase high-performance liquid chromatography (RP-HPLC, Fortis, C18 column, 250 mm × 4.6 mm internal diameter, pore size 5 μm, Waters system) was used to measure drug encapsulation efficiency. A mixture of acetonitrile/water in a ratio of 60:40 *v*/v with a flow rate of 1 ml min^{-1} was used as a mobile phase. The column effluent was monitored by a UV/VIS detector at 230 nm. The standard calibration curve of DTX in acetonitrile was created at 0.1–40 μg/ml. Next, 1 mg of freeze-dried micelles was dissolved in 50 μl DMSO (HPLC grade) to completely dissolve the co-polymer and break the polymer matrix. Thereafter, 5 ml of acetonitrile was added to dissolve DTX. The solution was then filtered by a syringe filter (0.45 μm PVDF membrane) for HPLC analysis and 40 μl of the obtained clear solution was injected into the column ($n = 3$) [23].

Study of *in vitro* release of DTX

The *in vitro* release of DTX from micelles was conducted by the dialysis method in a 0.01 mM phosphate buffer solution or PBS (pH 7.4) with 0.5% Tween 80. Next, 1 mg of each formulation was dispersed in PBS and placed in a dialysis membrane (6000–8000 Da) and dialyzed against an appropriate amount of PBS to maintain sink conditions. Following this, 1 ml of release medium was withdrawn at pre-determined time intervals and replaced with an equal volume of fresh medium. The concentration of DTX in the filtered sample was determined by the HPLC method ($n = 3$). After five days (120 h), the release efficiency (RE_{120}%) was determined for each formulation. Free DTX was formulated in Tween 80/ethanol/normal saline (20:13:67), which had the same formula as Taxotere®.

Tumor cell culture

The MC4-L2 murine positive GPER receptor breast cancer cell line [24] was cultured in DMEM-F12 media (supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 IU/ml penicillin) in T75 flasks in an oven appropriate for cell culture (5% CO_2 at 37 °C). Once the cells reached 75% confluence, they were washed and treated with trypsin to disconnect them from the culture plates and passaging.

Study of *in vitro* cell cytotoxicity

The MTT test was used to evaluate the cell toxicity of DTX-loaded micelles and free DTX using MC4-L2 cells. The cells were seeded in 96-well plates at a density of 10×10^3 cells per well and incubated for 24 h. Then the adherent cells were treated with different concentrations of free DTX, DTX-loaded and blank SMA-PAEEI-PEG, and SMA-PAEEI-PEG-RA micelles. After 48 h, the cells were treated with 20 μl/well of MTT solution (5 mg/ml in PBS) and incubated for a further four hours at 37 °C. Then the medium was removed and 150 μl of DMSO per well was added to dissolve formazan crystals. The absorbance of each well at 570 nm was measured using ELIZA reader (Awareness, US).

Animals

BALB/c mice (female, five to eight weeks old, weight: 20–25 g) were obtained from the Animal House at the School of Pharmacy (Isfahan University of Medical Science). All animals had a standard diet and all experiments were conducted according to the protocol of the Ethics Committee of Isfahan University of Medical Science.

In vivo pharmacokinetic study

BALB/c mice (female, weight: 20–25 g) were used to measure the pharmacokinetics of DTX-loaded micelles after IV injection. Mice were divided into two equal groups. One group received free DTX and another group received DTX-loaded RA-grafted micelles intravenously via the tail vein at a single dose of 7.5 mg/kg of DTX. Blood samples were taken from the femoral artery of the mice at five, 15, 20, and 30 min and one, two, four, six, and eight hours after drug injection; these blood samples were collected in heparin-impregnated tubes. Plasma samples were harvested by centrifugation at 10,000 rpm for 10 min and stored at −20 °C until analyzed for DTX [25].

HPLC method

The reverse-phase HPLC method and UV detection were used to measure the plasma concentrations of DTX. In a glass tube, 250 μl of plasma was poured and mixed for one minute with 25 μl of diazepam solution (25 μg/ml) as internal standard. Then plasma proteins were extracted by liquid-liquid extraction and 4 ml of diethyl ether. The mixture was centrifuged (Sigma 1–14, Germany) at 5000 rpm for 15 min, and the supernatant was collected and dried by nitrogen gas. Then the amount of DTX in dried residue (after being reconstituted with 200 μl of mobile phase) was determined by an HPLC system (Waters, USA) on a Bondapak C18 column (4.6 mm × 250 mm, 5 μm); the mobile phase, consisting of acetonitrile/water (60:40 *v*/v, flow rate of 1 ml min^{-1}) at 230 nm was determined by a UV detector. The amount of drug in the samples was then calculated by the standard calibration curve (0.1–10 μg/ml). The studied pharmacokinetic parameters included the area under the plasma concentration time curve from time zero to time infinity ($AUC_{0-\infty}$), clearance (CL), mean residence time from time zero to time infinity ($MRT_{0-\infty}$), volume of distribution at steady state (Vd_{ss}), elimination half-life ($T_{1/2\ \beta}$), and distribution half-life ($T_{1/2\ \alpha}$) [25].

Immunohistochemical examination

This test was done to assess the presence of GPER receptors in tumor tissues. After the euthanization of mice in each group, the tumors were immediately collected, frozen, fixed in 10% formalin, and embedded in paraffin blocks. One section was immune-stained using the GPER/GPR30 (AF5534) antibody and evaluated for the presence of GPER receptors in cancerous cells as compared to MDA-MB-231 as a GPER-negative breast cancer cell line.

Tumor induction

First, 2×10^6 MC4-L2 cells in 0.2 ml of PBS were immediately injected subcutaneously (*sc*) into the right flank in order to induce breast cancer model. The treatments were started when the tumor volume reached ~300 mm^3; this day was designated Day 0. The tumor diameter was measured by a caliper every other day in two dimensions and was calculated by the following equation:

$$V = \left(L \times W^2\right)/2$$

In the above, L is the largest and W is the smallest diameter.

In vivo antitumor activity

The mice were randomly divided into four equal groups (with five mice in each group). Each group received an equal dose of 7.5 mg/kg of the following formulations: (i) normal saline 0.9% as negative control; (ii) free DTX; (iii) DTX-loaded SMA-PAEEI-RA; and (iv) DTX-loaded SMA-PAEEI micelles through the tail vein every three days with a total of three doses. For 34 days after the first drug injection, the body weight and tumor growth of each mouse was measured every other day. After 34 days, the animals were sacrificed, and the tumor mass was harvested and weighed. The antitumor activity of each formulation was evaluated by the tumor inhibition rate (TIR%) using the following equation:

$$\mathrm{TIR}\% = \frac{\textit{Mean weight of tumor in negative control group} - \textit{Mean weight of tumor in treated group}}{\textit{Mean weight of tumor in negative control group}}$$

Survival study

Mice bearing MC4-L2 breast tumors (tumor size of ~300 mm^3) were divided into four groups, with five mice in each group, as follows: free DTX 7.5 mg/kg as positive control; normal saline as negative control; DTX-loaded SMA-PAEEI-PEG-RA 7.5 mg/kg as targeted group; and DTX-loaded SMA-PAEEI-PEG 7.5 mg/kg as non-targeted group. Three doses of the drug were administered intravenously for three days each and the mortality of the mice was monitored every day. The Kaplan–Meier plot was used to show the animal survival of breast cancer-bearing mice. The percentage increase in life span (%ILS) and the median survival time (MST) were calculated by the following equation [26]:

Median survival time (MST) = (day of 1st death + day of last death)/2% increase in life span = [(MST of treated group/ MST of control group) – 1] × 100.

Statistical analysis

Data (mean ± standard deviation) were analyzed by an independent sample t-test for two groups and a one-way analysis of variance (ANOVA) followed by a post hoc LSD test for multiple groups. $P < 0.05$ was considered statistically significant.

Results

Physicochemical characteristics of DTX-loaded micelles

The physicochemical properties of DTX-loaded SMA-PAEEI-PEG-RA and SMA-PAEEI-PEG were evaluated. These properties are shown in Table 1.

In vitro cell toxicity study

The MTT test was used to measure the effect of RA on the cytotoxicity of SMA-PAEEI-PEG micelles on MC4-L2 as a GPER-positive receptor cell line and was compared with free DTX cytotoxicity. In the MTT test, DTX-loaded polymeric micelles were prepared in deionized water in concentrations of 0.01, 0.1, 1, 10, and 100 nano-molar (nM) of DTX. Free DTX was dissolved in deionized water containing 1% DMSO that was diluted in culture media to 0.1%. Drug-free polymeric micelles were prepared at the same concentration of polymer that was used in drug-loaded micelles. In all concentrations, the cell viability percentage of blank micelles was more than the DTX-loaded micelles and free DTX ($p < 0.05$; Fig. 1). Targeted micelles (SMA-PAEEI-PEG-RA) loaded with DTX showed significantly more cytotoxicity ($p < 0.05$) in MC4-L2 cells as compared to free DTX, except at a concentration of 0.1 nM, and DTX-loaded non-targeted micelles (SMA-PAEEI-PEG), except at concentrations of 0.1 and 0.01 nM. There was no significant difference between the cytotoxicity of free DTX and DTX-loaded SMA-PAEEI-PEG micelles except at concentrations of 10 and 100 nM, that in these two concentrations, DTX-loaded SMA-PAEEI-PEG micelles were significantly more toxic than free DTX.

Pharmacokinetic studies

The plasma concentration time profiles (mean ± SD) of DTX in blood after mice are given *IV* injections of a single dose of 7.5 mg/kg of free DTX and DTX-loaded SMA-PAEEI-PEG-RA are shown in Fig. 2. The reduction in the plasma concentration following *iv* bolus delivery of the two formulations showed a two-compartment model. The related pharmacokinetic parameters are shown in Table 2, which were analyzed by an independent student's t-test. The DTX plasma concentration at five minutes after *iv* administration was 8.5 ± 0.31 μg/ml for the DTX-loaded SMA-PAEEI-PEG-RA micelles group that was higher (approximately 1.3 times) than that of the free DTX group, which was 6.5 ± 0.28 μg/ml.

As represented in Table 2, the AUC for SMA-PAEEI-PEG-RA micelles was approximately 1.3 times higher than that of free DTX (24.28 ± 3.3 μg.h/ml vs. 18.27 ± 1.2 μg.h/ml; $p < 0.05$). The elimination half-life ($T_{1/2\beta}$) of SMA-PAEEI-PEG-RA micelles (7.59 ± 0.24 h) was significantly ($p < 0.05$) longer than that of free DTX (6.33 ± 0.15 h). Moreover, the CL (total body clearance) and V_{dss} (steady state apparent volume of distribution) of SMA-PAEEI-PEG-RA micelles were significantly ($p < 0.05$) lower than those of free DTX. However, the MRT of the targeted micelles was more than that of free DTX but not significantly so ($p > 0.05$). Generally, the group which received SMA-PAEEI-PEG-RA micelles showed longer MRT, $T_{1/2\beta}$, and $T_{1/2\alpha}$, slower clearance (CL), and a smaller steady state volume distribution (V_{dss}) as compared to the free DTX group.

Immunohistochemical examination

The results of the immunohistochemical staining of excised tumor tissues by the GPER/GPR30 (AF5534) antibody and GPER-negative breast cancer cell line are shown in Fig. 3. The purple nucleus showed a negative stain and the absence of GPER receptor in the MDA-MB 231 cell line, which was used as the negative control (Fig. 3a); the brown nucleus showed a positive stain and the presence of GPER receptor in induced tumors by injection of the MC4-L2 cell line (Fig. 3b).

In vivo antitumor efficacy

About 10 days after the implantation of cancer cells, the tumors were well-developed (mean volume: ~300mm^3) and

Table 1 Physicochemical characteristic of docetaxel loaded targeted and non-targeted micelles (mean ± SD, n = 3)

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Release efficiency of 120 h (%)	CMC (μg/ml)
SMA-PAEEI-PEG-RA	128.5 ± 4.7	0.4 ± 0.0	−10.5	60.3 ± 2.0	89.7 ± 1.0	10.7
SMA-PAEEI-PEG	136.2 ± 2.2	0.3 ± 0.1	−6.9	54.1 ± 4.0	84.1 ± 2.9	29.8

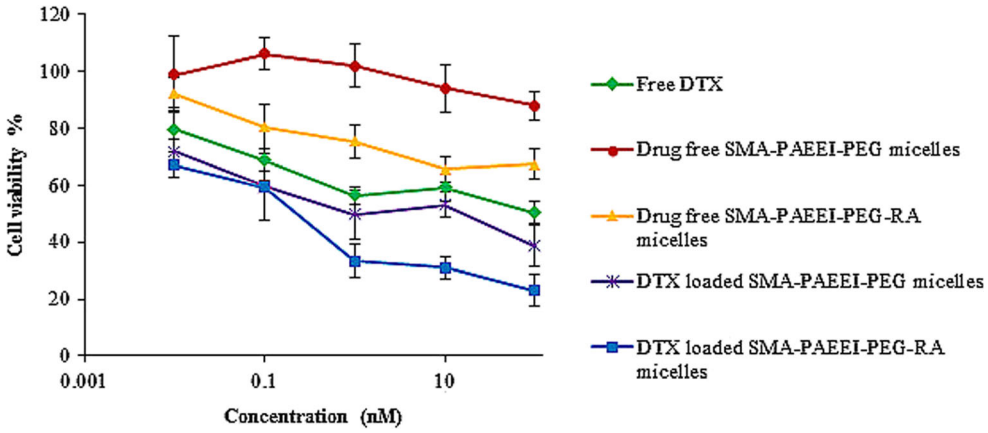

Fig. 1 Cell viability% of MC4-L2 cells (as GPER receptor positive cells) treated with free DTX, DTX loaded SMA-PAEEI-PEG-RA and SMA-PAEEI-PEG micelles, blank SMA-PAEEI-PEG-RA and SMA-PAEEI-PEG micelles

treatments were started. Free DTX and micellar solutions were injected at a dose of 7.5 mg/kg via the tail vein on days 0, 3, and 6. The safety and effectiveness of different formulations were compared by measuring the body weight and tumor volume of BALB/c mice after tumor implantation as shown in Fig. 4. As seen in Fig. 4a, the tumor volume in the normal saline group was excessively enlarged (4303.87 mm^3 after 45 days) as compared to DTX-encapsulating micelles and free DTX ($p < 0.05$). DTX-loaded SMA-PAEEI-PEG-RA micelles showed the least tumor volume (1241.23 mm^3) and demonstrated the greatest inhibitory effect of tumor growth as compared to other treatment groups ($p < 0.05$). As seen in Fig. 4b and Table 3, the body weight in the saline (negative control) group increased significantly up to ~18% during 45 days after the treatment due to fast tumor growth. However, in groups that received DTX-loaded micelles, a slight increase in body weight was observed after the drug treatment. Also, a slight decrease in body weight (~4%) was seen in the free DTX-treated group, which reflects the toxicity of the formulation.

At the end of this study, the weight of the excised tumor was recorded and shown in Fig. 5. As seen, DTX-loaded micelles and free DTX caused a marked inhibition of tumor size as compared to the saline control group ($p < 0.05$). By comparing the average end-point tumor volume in free DTX and DTX-loaded SMA-PAEEI-PEG and SMA-PAEEI-PEG-RA micelles groups, it was seen that micelles had significant antitumor efficacy as compared to free DTX ($p < 0.05$). DTX-loaded SMA-PAEEI-PEG-RA micelles demonstrated the least tumor weight as compared to free DTX and DTX-loaded non-targeted micelles ($p < 0.05$).

Table 3 shows the TIR of various treatment groups. For DTX-loaded RA-conjugated micelles, the TIR was 78.57%, which was significantly ($p < 0.05$) higher than the TIR values in other groups; this showed that DTX-loaded SMA-PAEEI-PEG-RA micelles could suppress tumor growth more efficiently than non-targeted DTX-loaded SMA-PAEEI-PEG micelles and free DTX.

Figure 6 shows the survival rate of MC4-L2 tumor-bearing BALB/c mice. Compared to the control (normal saline) groups, the survival of mice in free DTX-treated groups or in DTX-loaded micelles groups was higher. As shown in Table 3, the median survival time (MST) after the *iv* injection of three doses of 7.5 mg/kg in mice treated with normal saline, free DTX, and DTX-loaded SMA-PAEEI-PEG micelles was 12, 17, and 20 days, respectively. However, the MST of mice treated with DTX-encapsulated SMA-PAEEI-PEG-RA micelles was greater than 22 days and longer than that of the other treatment groups. The percentage increase in lifespan

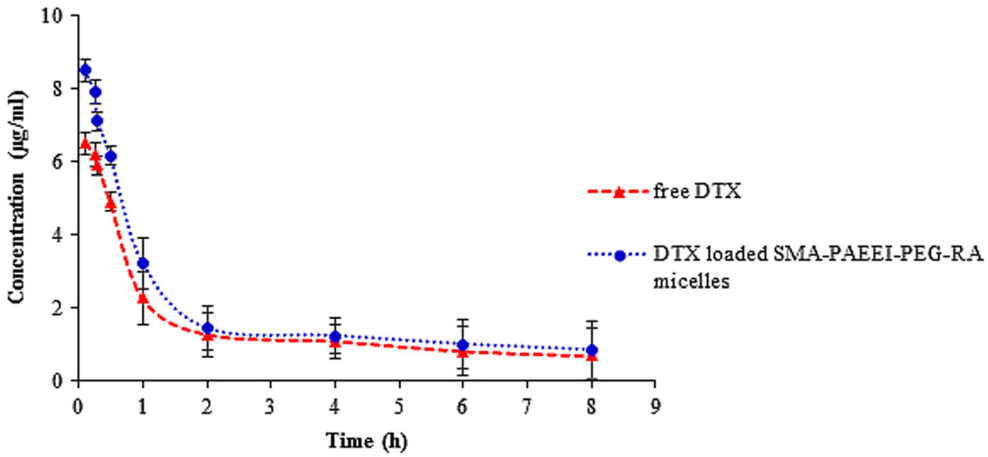

Fig. 2 The mean plasma-concentration versus time profiles of free DTX and DTX loaded SMA-PAEEI-PEG-RA micelles (mean ± SD, $n = 3$)

Table 2 The pharmacokinetic parameters of DTX loaded SMA-PAEEI-PEG-RA micelles and free DTX calculated from plasma samples

Formulations	$AUC_{0-\infty}$ (μg.h/ml)	CL(ml/kg.h)	$MRT_{0-\infty}$ (h)	Vd_{ss}(L/kg)	$T_{1/2\ \beta}$ (h)	$T_{1/2\ \alpha}$ (h)
Free DTX	18.27 ± 1.20	410.43 ± 42.15	7.38 ± 0.87	3.75 ± 0.44	6.33 ± 0.15	0.33 ± 0.016
DTX-loaded SMA-PAEEI-PEG-RA	24.28 ± 3.3	308.80 ± 31.33	8.66 ± 0.75	2.67 ± 0.28	7.59 ± 0.24	0.43 ± 0.001

Data is shown as mean ± SD (n = 3). $AUC_{0-\infty}$: the area under the plasma concentration–time curve from time zero to time infinity, CL: total body clearance, $MRT_{0-\infty}$, mean residence time from time zero to time infinity, V_{dss}: steady state volume of distribution, $T_{1/2\beta}$: apparent plasma half-life of elimination phase, $T_{1/2\alpha}$: apparent plasma half-life of distribution phase

(%ILS) of mice treated with free DTX and DTX-loaded SMA-PAEEI-PEG micelles was 41.66% and 66.66%, while the %ILS of DTX-loaded SMA-PAEEI-PEG-RA micelles-treated mice exceeded 83.33%.

Discussion

Despite the abundant use of DTX, a semi-synthetic analog of paclitaxel (used for the treatment of several types of cancer including ovarian, breast, and non-small cell lung cancer) has some toxic effects [27] due to the presence of excipients in solvent-base formulations that cause hypersensitive responses. These *iv* formulations containing dissolved and molecular DTX deliver drugs non-specifically through the whole body (tumors and also normal tissues) [28]. Additionally, the non-specific systemic delivery of cytotoxic drugs leads to poor therapeutic outcomes and dose-dependent side effects [29]. To overcome these restrictions, many nano polymeric-based drug delivery systems have been prepared. Polymeric micelles are one of the tumor-targeting nanocarrier drug delivery systems, which are formed from the self-assembly of amphiphilic block copolymers in an aqueous medium. They consist of a hydrophobic reservoir core for loading of drugs and a hydrophilic shell (like PEG) for stabilization of the micelle. Polymeric micelles also deliver drugs specifically to tumor tissues, enhance drug accumulation in tumors, improve its bio-distribution, and have lower cytotoxicity and side effects on normal tissues as compared to free chemotherapeutic drugs [30]. Based on the present study, a novel polymeric nanomicelle composed of SMA as a core-forming polymer and PAEEI-PEG as a hydrophilic shell targeted by RA (ligand for GPER receptors) was synthesized for the delivery of DTX. The size of targeted and non-targeted nanomicelles was 128.5 ± 4.7 nm and 136.2 ± 2.2 nm, respectively, which can enter and accumulate within the interstitial space of tumor sites. As shown in Fig. 1, the designed non-target polymer showed no toxicity, and the blank micelles of both targeted and non-targeted micelles had lower toxicity than DTX-loaded ones and free DTX. The greater cytotoxicity of DTX-loaded SMA-PAEEI-PEG-RA micelles in MC4-L2 cells is possibly because of the increase in the cellular uptake of the micelles by GPER receptor mediated endocytosis, which is in accordance with *in vivo* antitumor results (Figs. 4 and 5). These results are similar to the report by Varshosaz et al. [25] that shows a higher toxicity of DTX-loaded folic acid-targeted micelles against folate positive melanoma (B16F10) cells as compared to non-targeted micelles; the study concluded that this was due to the folate receptor endocytosis-mediated entrance of nanomicelles. As seen in Fig. 2, the DTX concentration after the injection of DTX was at the highest level and rapidly reduced during the first hour of administration. After the *iv* injection of nanomicelles, the entrapped drug in the micelles was slowly released from the core of the micelles and, therefore, not distributed readily; this led to a higher drug concentration at the time of first sampling as compared to the free drug. The concentration of DTX for DTX-loaded nanomicelles at the first sampling time (five minutes) was approximately 1.3 times higher than

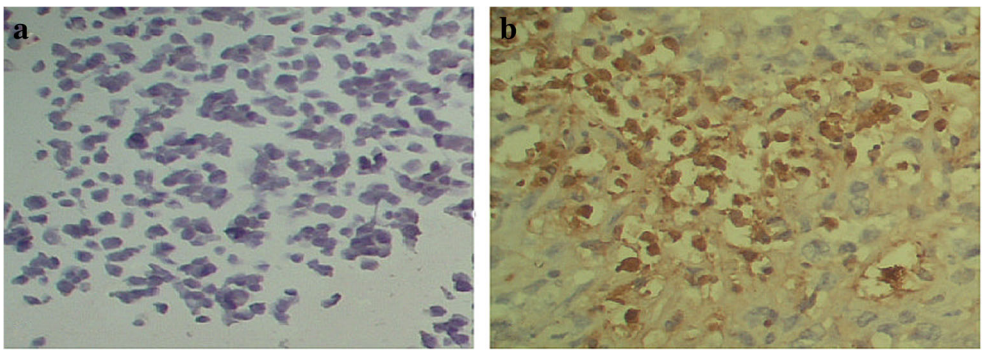

Fig. 3 Immunohistochemical staining of **a)** MDA-MB-231 human GPER negative breast cancer cells and **b)** breast cancer tumors induced by MC4-L2 cells by GPER/GPR30 (AF5534) antibody

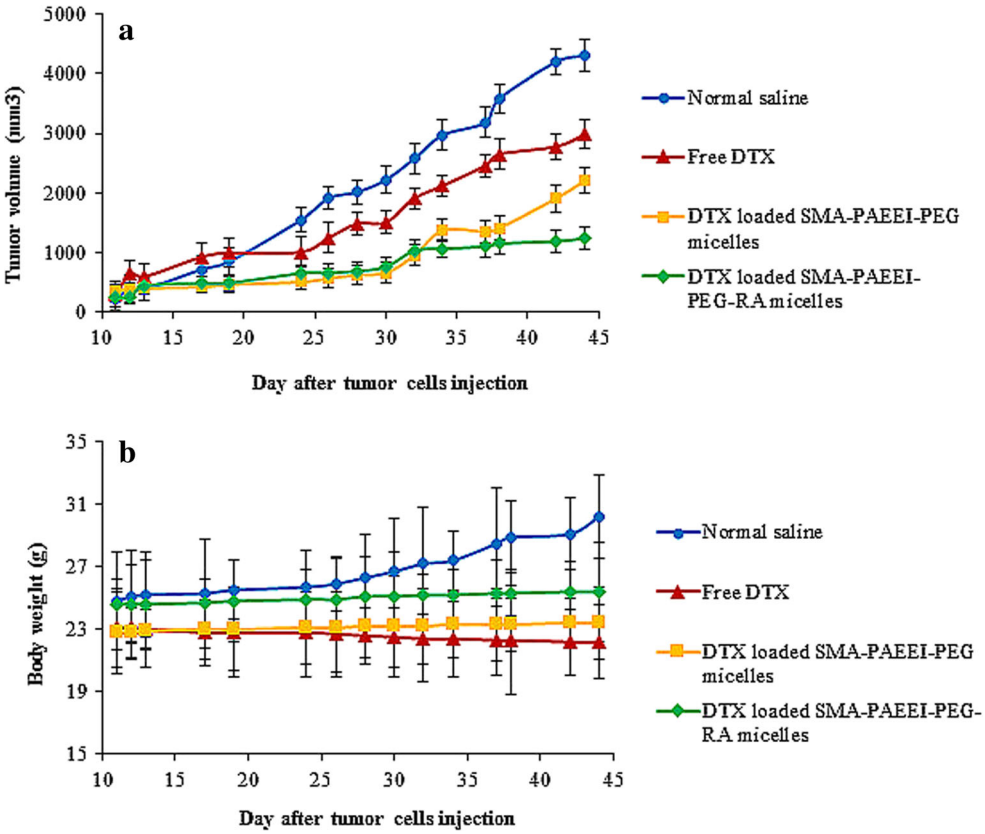

Fig. 4 *In vivo* antitumor activity of free DTX and DTX loaded in targeted and non-targeted micelles in BALB-c mice bearing breast cancer by showing **a)** tumor growth (tumor volume (mm^3) and **b)** body weight (g) during the study

the concentration of free DTX at the same time. These results are in agreement with the findings of Zhao et al. [31] who reported a higher DTX concentration at the time of the first sampling after the *iv* injection of a liposomal DTX formulation as compared to the free DTX injection. Different studies have indicated that loading chemotherapeutic drugs inside the core of micelles led to changes in the pharmacokinetic parameters, including reduced renal clearance, prolonged circulation time (increased $T_{1/2\beta}$), higher AUC (area under the concentration-time curve), and higher MRT (mean residence time) as compared to free chemotherapeutic drugs [28, 30, 32]. Increments of AUC, MRT, and $T_{1/2\beta}$ of DTX in SMA-PAEEI-PEG-RA micelles were also shown in our study (Table 2), which together can reduce DTX elimination time and increase blood circulation time [25]. This long circulation property and high stability of SMA-PAEEI-PEG-RA micelles led to selective accumulation of micelles in tumor tissues and high intracellular concentration of drugs [33]. The long blood circulation of micelles can be attributed to the stealth behavior of SMA-PAEEI-PEG-RA micelles induced by the hydrophilic nature of the PEG shell. The steric hindrance and hydration of PEG chains in a PEGylated polymer in an aqueous environment improved the stability of the drug delivery system in the blood by preventing drug adsorption on the surface of the plasma protein (opsonization) and uptake by reticuloendothelial systems (RES) [34]. DTX-loaded SMA-PAEEI-PEG-RA

Table 3 MST (median survival time), TIR (tumor inhibition ratio), %ILS (percentage increase in life span) and changing in body weight of breast cancer bearing BALB-c mice in different treatment group (normal saline, DTX loaded targeted and non-targeted micelles formulations and free DTX)

Test groups	MST (day)	TIR (%)	PILS (%)	Body weight (g)	
				Initial	Final
Normal saline	12	……	…….	24.80 ± 3.16	30.20 ± 4.34
DTX loaded SMA-PAEEI-PEG-RA	> 22	78.57%	>83.33%	24.66 ± 1.63	25.46 ± 3.26
DTX loaded SMA-PAEEI-PEG	20	57.14%	66.66%	22.82 ± 2.36	23.42 ± 3.89
Free DTX	17	51.19%	41.66%	23.14 ± 3.06	22.24 ± 2.45

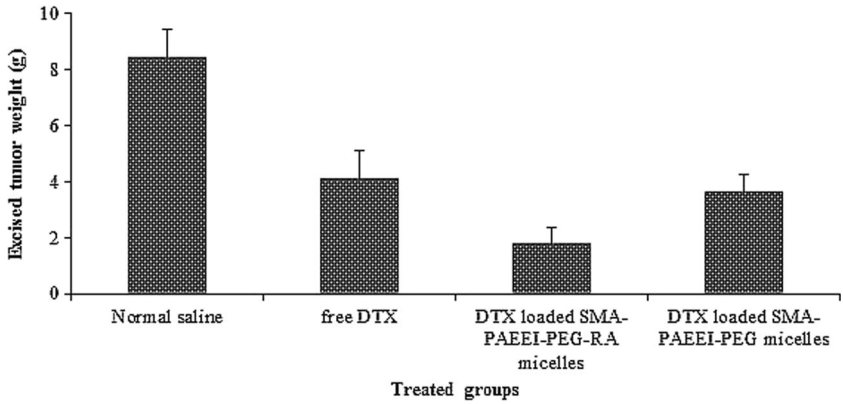

Fig. 5 Excised tumor's weigh at the end of study ($n = 5$)

polymeric nanomicelles demonstrated higher AUC ($p < 0.05$), MRT ($p > 0.05$), and $T_{1/2\beta}$ ($p < 0.05$), and lower CL ($p < 0.05$) and V_{dss} ($p < 0.05$) as compared to free DTX. To assess the antitumor effects of chemotherapeutic drugs on human tumors, researchers use immune-deficient animal models of malignant diseases [35] but a healthy immune system is important to fight cancer cells and is necessary for a comparison with healthy immune systems of humans [36]. Therefore, in the present study, murine MC4-L2 breast cancer cells were used to induce positive GPER receptor breast tumor in BALB/c mice; thereafter, *in vivo* antitumor activity of free DTX, DTX-loaded non-targeted SMA-PAEEI-PEG, and targeted SMA-PAEEI-PEG-RA micelles were investigated (Fig. 4). The body weight, tumor volume, TIR, %ILS, and MST of BALB/c mice after tumor implantation were used to assess the safety and effectiveness of different formulations. In the group receiving free DTX, the initial treatment decreased the tumor growth rate but later the tumor volume grew more than in the other groups. As shown in Figs. 4 and 5, the variation in the body weight of animals was monitored during 45 days as an index for the adverse effects and systemic toxicity of the formulations. The decrease in body weight in mice receiving free DTX is because of non-specific distribution and more toxicity effects on normal tissues [29]. Another reason for the greater efficacy of micelles as compared to free drugs may be due to the accumulation of DTX-loaded SMA-PAEEI-PEG-RA micelles into tumor tissues after binding with the GPER receptor, a member of the estrogen receptor family [17], in estrogen-positive breast cancer cells and internalization by endocytosis; this leads to the intracellular delivery of DTX, which may contribute to the enhanced efficacy of targeted micelles. However, in non-targeted micelles, drug distribution and release in the extracellular space by the EPR effect results in the loss of its effect [36, 37]. Generally, these results showed greater antitumor activity of DTX-loaded RA-targeted micelles in MC4-L2 tumor-bearing mice, which could be due to the transport of DTX into the tumor tissues more efficiently.

Suppression of tumor growth was shown by TIR and the survival rate was shown by MST, %ILS, and the Kaplan–Meier curve (Table 3 and Fig. 6). In groups receiving DTX-loaded RA-targeted micelles, TIR, MST, and %ILS were significantly higher than in free DTX groups ($p < 0.05$); this is due to better inhibitory effects of DTX-loaded SMA-PAEEI-PEG-RA micelles on tumor growth and more survival effects of targeted micelles on tumor-bearing mice. These findings are in agreement with the study by Varshosaz et al. [25], which showed that folic acid (FA)-targeted PF127-Chol micelles significantly "inhibited tumor growth compared to Taxotere® and DTX-loaded non-targeted micelles."

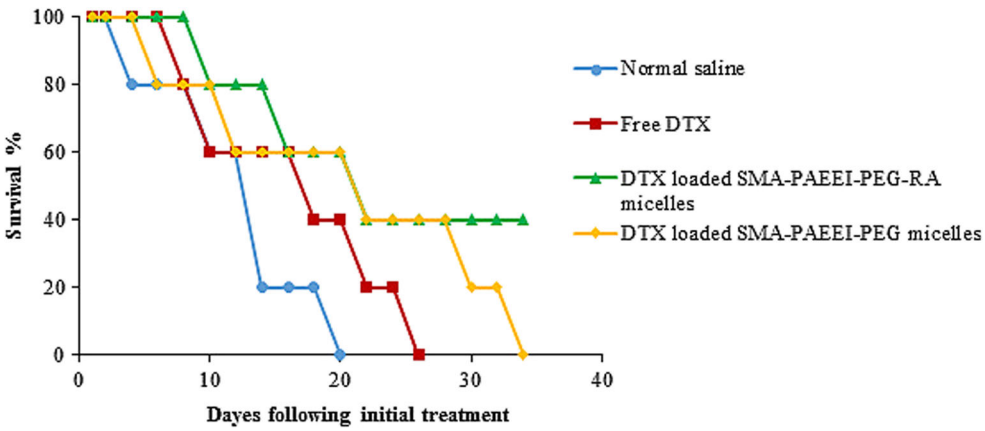

Fig. 6 Kaplan-Meier survival curves of MC4-L2 tumor-bearing BALB-c mice that treated with DTX loaded SMA-PAEEI-PEG-RA micelles, DTX loaded SMA-PAEEI-PEG micelles, free DTX and normal saline

Conclusion

The novel drug delivery carrier targeted by RA that conjugated to SMA-PAEEI-PEG co-polymeric micelles encapsulating DTX showed decreased cell viability and increased $AUC_{0-\infty}$, reduced $V_{dss,}$ and prolonged MRT as compared to free DTX. Treatment with DTX-loaded SMA-PAEEI-PEG-RA micelles indicated more antitumor activity (TIR) and increased the survival rate of mice (MST) as compared to other treated groups. These pharmacokinetic behaviors and antitumor efficiency in cancerous mice illustrated the potential suitability of SMA-PAEEI-PEG-RA micelles for the targeted delivery of hydrophobic anticancer drugs in the treatment of GPER-positive breast tumor with a long circulating time and low toxicity. These obtained results suggest that nanomicelles targeted by RA may be a promising approach to achieve tumor-specific drug delivery.

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Compliance with ethical standards

Conflict of interest Saeede Enteshari declares that she has no conflict of interest. Jaleh Varshosaz declares that she has no conflict of interest. Mohsen Minayian declares that he has no conflict of interest. Farshid Hassanzadeh declares that he has no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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