International Journal of Pharmaceutics 431 (2012) 183–189

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

# The *in vivo* antitumor activity of LHRH targeted methotrexate–human serum albumin nanoparticles in 4T1 tumor-bearing Balb/c mice

Azade Taheri [a,b], Rassoul Dinarvand [a,c,*], Fatemeh Ahadi [a], Mohammad Reza Khorramizadeh [d], Fatemeh Atyabi [a,c]

[a] Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[b] Department of Pharmaceutics, Faculty of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran
[c] Nanotechnology Research Centre, Faculty of Pharmacy, Tehran University of Medical sciences, Tehran, Iran
[d] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

*Article history:*
Received 8 February 2012
Received in revised form 6 April 2012
Accepted 8 April 2012
Available online 15 April 2012

*Keywords:*
Nanoparticles
Methotrexate
Human serum albumin
LHRH
*In vivo* tumor targeting

ABSTRACT

The use of targeted drug delivery systems is a growing trend in cancer treatment to decrease the adverse effect of anti-cancer drugs. In this study, we sought to conjugate methotrexate–human serum albumin nanoparticles (MTX–HSA NPs) with luteinizing-hormone releasing hormone (LHRH). The LHRH was intended to target LHRH receptors overexpressed on the several types of tumors. The expression of LHRH receptors on the 4T1 breast cancer cells was confirmed by FITC conjugated LHRH receptor antibody using fluorescence microscopy. Female Balb/c mice bearing 4T1 breast cancer tumor were treated with a single i.v. injection of free MTX, non-targeted MTX–HSA NPs and LHRH targeted MTX–HSA NPs. LHRH targeted MTX–HSA nanoparticles showed stronger anti-tumor activity *in vivo*. By 7 days after treatment, average tumor volume in the LHRH targeted MTX–HSA NPs treated group decreased to 8.67% of the initial tumor volume when the number of attached LHRH molecules on MTX–HSA NPs was the highest, while the average tumor volume in non-targeted MTX–HSA NPs treated mice grew rapidly and reached 250.7% of the initial tumor volume 7 days after the treatment. LHRH targeted MTX–HSA NPs could significantly extend the survival time of tumor bearing mice compared with the non-targeted MTX–HSA NPs and free MTX formulations.



## 1. Introduction

Chemotherapy is one of the strategies for the treatment of different types of cancers. However, the toxic side effects of the effective doses of chemotherapeutic agents could restrict the use of these agents. In recent years researchers have focused on the use of the targeted drug delivery systems to produce effective anti-cancer therapies with fewer side effects (Dinarvand et al., 2011; Jin et al., 2011). Active tumor targeting of drugs could be achieved using surface modification of nanosized drug delivery systems with targeting moieties. Targeting moieties could specifically direct the delivery system to specific binding sites on the cancer cells (Esmaeili et al., 2008; Minko, 2004). Several types of component such as sugars (David et al., 2004; Krishnaiah et al., 2002), vitamins (Yang et al., 2009), folate (Zhao et al., 2010; Gupta et al., 2010) and antibodies (Lukyanov et al., 2004) have been used as targeting moieties in targeted drug delivery systems. Among peptides, luteinizing hormone releasing hormone (LHRH) could be used as an effective targeting moiety in targeted drug delivery systems for several types of cancers (Dharap et al., 2005; He et al., 2010). LHRH, a decapeptide produced in the hypothalamus, plays an important role in the hormonal control of the reproductive system. LHRH receptors are overexpressed in several types of cancerous cells such as breast, ovarian, and prostate cancer cells (Reubi, 2003; Schally and Nagy, 2003; Leuschner and Hansel, 2005).

In our previous works the preparation of MTX–HAS conjugated nanoparticles and their surface modification with LHRH as a targeting moiety to design a tumor targeted drug delivery system based on human serum albumin (HSA) conjugation strategy have been reported (Taheri et al., 2011a,b). The LHRH targeted drug delivery system consisted of (i) nanoparticles of human serum albumin (HSA) as carrier; (ii) methotrexate (MTX) as anticancer drug; and (iii) LHRH as a targeting moiety. The targeting effect of LHRH targeted MTX–HSA NPs to tumor cells were confirmed successfully *in vitro* (Taheri et al., 2011b).

The 4T1 breast cancer cells are derived from spontaneously Balb/c mammary carcinoma (Tao et al., 2008). 4T1 is a transplantable tumor cell line that its metastatic and invasive properties are well documented (Heppner et al., 2000). The 4T1 breast cancer

* Corresponding author at: Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 1417614411, Iran. Tel.: +98 21 66959095; fax: +98 21 66959096.
*E-mail address:* dinarvand@tums.ac.ir (R. Dinarvand).

0378-5173/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijpharm.2012.04.033

closely resembles metastatic breast cancer in human patients and could be used as a suitable model for evaluation of the efficacy of anticancer drugs such as MTX efficiently (Aslakson and Miller, 1992).

In this study, we firstly confirmed the expression of LHRH receptors on 4T1 breast tumor cells by FITC labeled LHRH receptor antibody using fluorescence microscopy, thus 4T1breast tumor could be used as a model for evaluation of LHRH targeted drug delivery systems. Then in order to increase the anti-tumor efficacy of MTX, the feasibility of using LHRH as a targeting moiety to target MTX–HSA NPs to the 4T1 breast tumor cells *in vivo* was studied.

## 2. Materials and methods

### 2.1. Materials

LHRH, HSA, EDC, N-hydroxy succinimide were all purchased from Sigma (Steinheim, Germany). Methotrexate USP was kindly donated by Cipla Pharmaceutical Co, India. Total protein kit (Micro Lowry) was from Sigma (Saint Louis, USA). FITC labeled LHRH receptor antibody was obtained from Biorbyt (Cambridge, UK). T47D, a human breast cancer cell line, SKOV3, a human ovarian cancer cell line and breast tumor 4T1 cell lines were obtained from American Type Culture Collection (Manassas, VA). RPMI-1640 modified medium and penicillin/streptomycin solution was obtained from Gibco Invitrogen (Calsbad, CA). All other reagents were of analytical grade. Deionized water was used throughout the experiment.

### 2.2. Preparation of LHRH targeted MTX–HSA NPs

MTX was conjugated to HSA by a carbodiimide reaction using EDC and NHS (Taheri et al., 2011a). Then MTX–HSA conjugates were cross-linked using EDC to form MTX–HSA NPs. MTX/HSA molar ratio in MTX–HSA NPs was determined (Taheri et al., 2011a). In this study, we used MTX–HSA NPs with MTX/HSA molarity ratio of $8 \pm 0.18$. For conjugation of LHRH molecules on the surface of MTX–HSA NPs, 2, 5 and 10 mg of LHRH were added to MTX–HSA NPs (MTX/HSA molar ratio: 8) (25 mg in 1 mL water) and mixed 250 μL of a freshly prepared EDC solution (10 mg in 1 mL of water) was added to LHRH and MTX–HSA NPs mixture, and the solution was maintained at 4 °C for 15 h. Unreacted EDC and LHRH were removed using Amicon® Ultra-4 Centrifugal Filter Devices with cutoff of 30 kDa (Millipore, USA). Total protein kit (Micro Lowry) was used for the determination of the amount of LHRH coupled to MTX–HSA nanoparticles. The total amount of LHRH bound to the MTX–HSA nanoparticles was calculated as the difference between the total amount of LHRH used for conjugation and the amount of unreacted LHRH determined after the filtration step described above.

Particle size, zeta potential, stability, *in vitro* cytotoxicity and cellular uptake of LHRH targeted MTX–HSA NPs were determined in our previous works (Taheri et al., 2011a,b).

### 2.3. Cell culture

Breast tumor 4T1 cell line, human breast cancer cells (T47D) and ovary cancer cells (SKOV3) from American Type Culture Collection (Manassas, VA), cultured in RPMI 1640 that supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin.

### 2.4. Evaluation of the expression of the LHRH receptor on 4T1 tumor cells

The expression of the LHRH receptors has been studied on 4T1breast cancer cells *in vitro*. Primary cultures of 4T1 breast cancer cells, T47D breast cancer cells (LHRH receptor positive cell (Günthert et al., 2004)) and SKOV3 tumor cells (LHRH receptor negative cell (Taratula et al., 2009)) were prepared in 6-well plates (Costar, IL, USA) at a density of 300,000 cells per well. FITC labeled LHRH antibody (10 μg antibody/$10^6$ cells) was directly added to cells and incubated for 45 min at 37 °C. After 45 min, the cells were then thoroughly washed three times with PBS (pH 7.4) and evaluated by fluorescence microscopy (Olympus IX 71, Japan).

### 2.5. Tumor inoculation

All animal experiments were done in accordance with protocols approved by the ethical committee of the Pharmaceutical Research Centre, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran. The female Balb/c mice with the weight of 17–20 g (6–8 weeks old, Pasteur Institute, Tehran, Iran) were provided and maintained on free access to food and water. Then female Balb/c mice were injected subcutaneously with 4T1 breast cancer cell solution (0.2 mL) in the breast region with $10^5$–$10^6$ cells. When the tumor sizes of 95% of tumor bearing mice were greater than $170 \pm 89\ mm^3$, the mice were divided into 11 groups. There were 22 mice in control group (normal saline treated group) and there were 7 mice in each other groups. 5–6 mice that had approximately same tumor size were placed in one group. Tumor length and width were measured using a digital caliper and calculated using the following formula:

$$0.4 \quad (L \cdot W^2)$$

where $L$ is the length and $W$ is the width of the tumors (Vredenburg et al., 2001).

Prior to treatment, all the mice were numbered and weighed, moreover the initial tumor volumes were recorded. Test animals received a single i.v. injection *via* the tail vein of LHRH targeted MTX–HSA NPs, non-targeted MTX–HSA NPs and free MTX formulation at MTX dose of 6.25 and 12.5 mg/kg (around 0.2 mL). The control animals received an injection of 0.2 mL of normal saline. The tumor volume and weight of each mouse were measured over a period of 21 days. Eleven animal groups were used for the *in vivo* study as below.

Group 1: saline treated group (22 mice); Group 2: MTX (6.25 mg/kg) treated group (7 mice); Group 3: MTX (12.5 mg/kg) treated group (7 mice); Group 4: MTX–HSA NPs (equivalent 6.25 mg/kg free MTX) treated group (7 mice); Group 5: MTX–HSA NPs (equivalent 12.5 mg/kg free MTX) treated group (7 mice); Group 6: LHRH 5.8–MTX–HSA NPs (equivalent 6.25 mg/kg free MTX) treated group (7 mice); Group 7: LHRH 5.8–MTX–HSA NPs (equivalent 12.5 mg/kg free MTX) treated group (7 mice); Group 8: LHRH 17.3–MTX–HSA NPs (equivalent 6.25 mg/kg free MTX) treated group (7 mice); Group 9: LHRH 17.3–MTX–HSA NPs (equivalent 12.5 mg/kg free MTX) treated group (7 mice); Group 10: LHRH 29.1–MTX–HSA NPs (equivalent 6.25 mg/kg free MTX) treated group (7 mice); Group 11: LHRH 29.1–MTX–HSA NPs (equivalent 12.5 mg/kg free MTX) treated group (7 mice).

### 2.6. Determination of median survival time and percentage increase in life span

The mortality was monitored by recording the median survival time (MST) and percentage increase in life span (ILS%), determined by the following formulae.

$$\mathrm{MST} = \frac{\text{Day of 1st death} + \text{Day of last death}}{2}$$

$$\%\mathrm{ILS} = [(\text{MST of treated group}/\text{MST of control group}) - 1] \times 100$$

(Geran et al., 1972).

### 2.7. Evaluation of body weight loss

The anti-tumor efficacy of LHRH targeted MTX–HSA NPs could be evaluated by inhibitory effects of these targeted nanoparticles on the weight loss of tumor bearing mice (Tseng et al., 2009). Consequently the body weights of 4T1 tumor bearing mice treated with free MTX, non-targeted MTX–HSA NPs and LHRH targeted MTX–HSA NPs were recorded simultaneously every 3 days during the study.

### 2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Preparation of LHRH targeted MTX–HSA nanoparticles

LHRH targeted MTX–HSA NPs were prepared using three different LHRH/HSA molar ratios namely $5.8 \pm 0.12$ (as LHRH 5.8–MTX–HSA NPs), $17.3 \pm 0.23$ (as LHRH 17.3–MTX–HSA NPs) and $29.1 \pm 0.2$ (as LHRH 29.1–MTX–HSA NPs). The results of the characteristics of LHRH targeted MTX–HSA NPs are presented in Table 1. The *in vitro* cytotoxicity and cellular uptake of LHRH targeted MTX–HSA NPs were evaluated on LHRH receptor positive T47D cells and LHRH receptor negative SKOV3 cells in our previous study (Taheri et al., 2011b). Briefly, the cytotoxicity of the LHRH targeted MTX–HSA NPs on the LHRH receptor positive T47D tumor cells were significantly higher than non-targeted MTX–HSA NPs. LHRH targeted nanoparticles were also internalized by LHRH receptor positive T47D cells significantly more than non-targeted nanoparticles. There were no significant differences between the uptake of LHRH targeted and non-targeted MTX–HSA NPs in the LHRH receptor negative SKOV3 cells. The active targeting procedure using LHRH targeted MTX–HSA NPs could increase the anti-tumoral efficacy of MTX. In addition the cytotoxicity and cellular uptake of LHRH targeted MTX–HSA NPs on the LHRH receptor positive T47D cells was increased proportionate to the number of attached LHRH molecules on the surface of MTX–HSA NPs.

### 3.2. Evaluation of the expression of the LHRH receptor on 4T1 tumor cells

The binding of FITC-labeled LHRH receptor antibodies on the surface of LHRH receptor positive cells could exhibit membrane fluorescence. As shown in Fig. 1a, T47D LHRH receptor positive cells exhibited membrane fluorescence after 45 min of incubation with FITC-labeled LHRH receptor antibody at 37 °C. But, SKOV3 LHRH receptor negative cells did not show any fluorescence when incubated with FITC-labeled LHRH receptor antibodies (Fig. 1b). Similar to T47D LHRH receptor positive, 4T1 tumor cells exhibited membrane fluorescence after incubation with FITC-labeled LHRH receptor antibody at 37 °C (Fig. 1c). Thus the membrane fluorescence exhibition of 4T1 tumor cells after incubation with FITC-labeled LHRH receptor antibody could confirm the expression of LHRH receptors on the surface of 4T1 tumor cells.

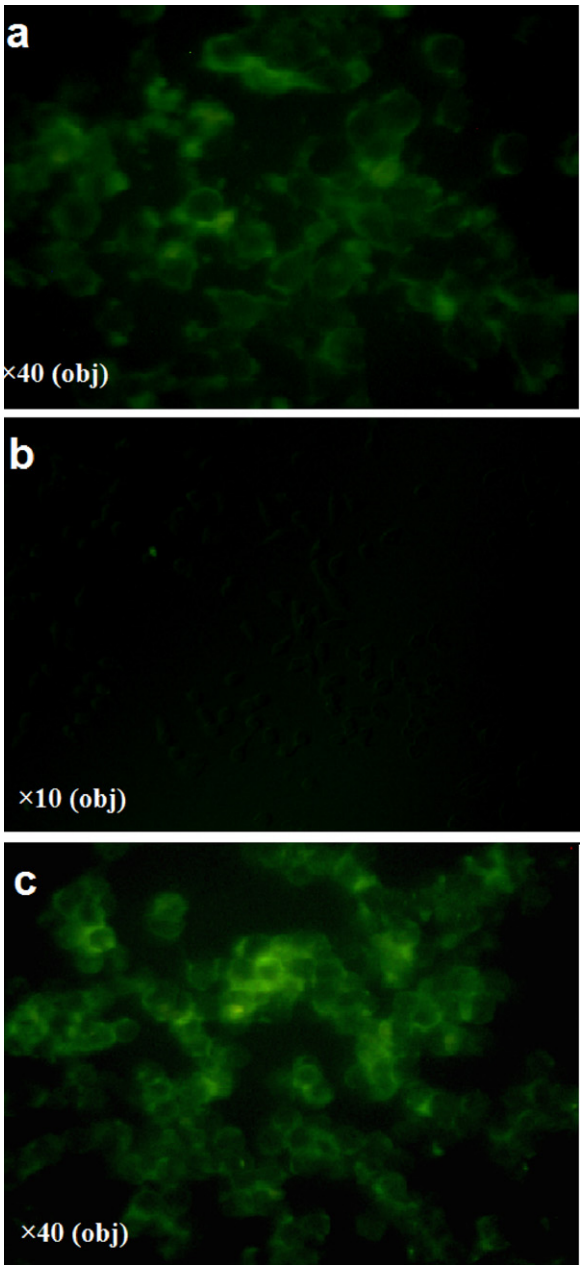


**Fig. 1.** Fluorescence microscopy images of (a) T47D, (b) SKOV3 and (c) 4T1 tumor cells after incubation with FITC labeled LHRH receptor antibody for 45 min at 37 °C.

### 3.3. In vivo anticancer efficacy of LHRH targeted nanoparticles

Fig. 2 shows the growth of 4T1 breast tumors as a function of times after treatment with two different dose of free MTX (6.25 and 12.5 mg/kg), non-targeted MTX–HSA NPs (equivalent to 6.25 and 12.5 mg/kg free MTX) and LHRH 17.3–MTX–HSA NPs (equivalent to 6.25 and 12.5 mg/kg free MTX). At the doses used (equivalent to 6.25 and 12 mg/kg free MTX), free MTX did not have

**Table 1**
Physicochemical characteristics of MTX–HSA NPs (MTX/HSA molar ratio: 8) and LHRH targeted MTX–HSA NPs (mean ± SD; $n = 3$).

| | Non targeted MTX–HSA NPs | LHRH 5.8–MTX–HSA NPs | LHRH 17.3–MTX–HSA NPs | LHRH 29.1–MTX–HSA NPs |
|---|---|---|---|---|
| Particle size (nm) | 111.7 ± 4.6 | 120.5 ± 2.7 | 128.45 ± 4.40 | 138.56 ± 3.20 |
| Poly dispersity | 0.10 ± 0.01 | 0.12 ± 0.09 | 0.20 ± 0.04 | 0.14 ± 0.05 |
| Zeta potential (mV) | −12.10 ± 0.50 | −10.45 ± 1.23 | −10.10 ± 1.10 | −10.04 ± 0.65 |
| LHRH/HSA molar ratio | – | 5.80 ± 0.12 | 17.30 ± 0.23 | 29.10 ± 0.20 |

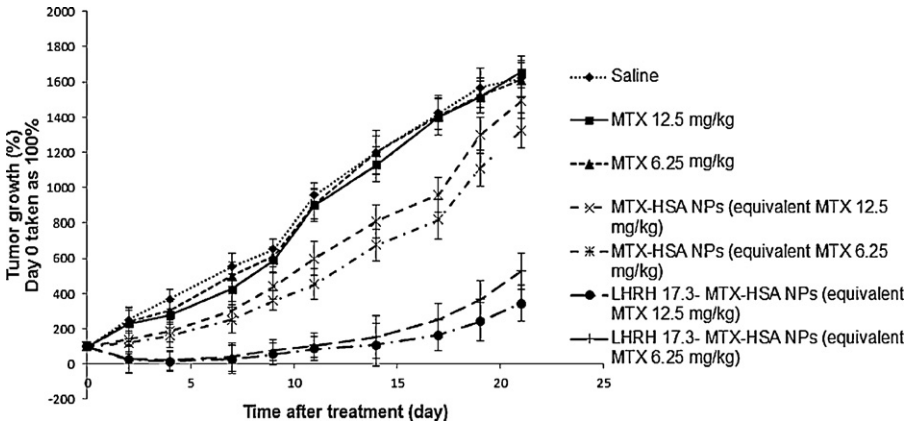


**Fig. 2.** Antitumor effect of free MTX, MTX–HSA NPs and LHRH 17.3–MTX–HSA NPs on 4T1 tumor bearing mice. 4T1 tumor cells were implanted s.c. in Balb/c mice. The drugs were injected i.v. in a single dose (day 0). The doses were equivalent to 6.25 and 12.5 mg/kg of free MTX. Data are presented as mean ± SD of relative tumor volumes (day 0 taken as 100%).

significant inhibitory effect on tumor growth compared to control group (saline treated group). The inhibitory tumor growth effect of non-targeted MTX–HSA NPs was only 1.54 to 1.98-fold more than free MTX at similar concentration. As seen clearly in Fig. 2, LHRH 17.3–MTX–HSA NPs treated groups (equivalent 6.25 and 12.5 mg/kg free MTX) exhibited stronger anti-tumor effect to the same dose of MTX–HSA NPs treated group. The tumor volume of the mice received LHRH 17.3–MTX–HSA NPs (equivalent to 12.5 mg/kg free MTX) was 6.15 ± 0.98 more suppressed than tumor volume in the non-targeted MTX–HSA NPs (equivalent 12.5 mg/kg free MTX) treated group on day 14 after treatment. The lower dose of LHRH 17.3–MTX–HSA NPs (equivalent 6.25 mg/kg free MTX) was also effective in inhibiting tumor growth. 14 days after treatment, the tumor volume of the mice treated with LHRH 17.3–MTX–HSA NPs (equivalent 6.25 mg/kg free MTX) was 5.23 ± 0.45 and 4.38 ± 0.56-fold lower than those treated with non-targeted MTX–HSA NPs (equivalent to 6.25 mg/kg free MTX) and those treated with non-targeted MTX–HSA NPs (equivalent to 12.5 mg/kg free MTX). As shown in Fig. 2, the significant anti-tumor effect in mice treated with LHRH targeted MTX–HSA NPs was observed from day 4 up to day 21 of treatment ($p < 0.05$).

Moreover, the results of *in vivo* anti-tumor effect of LHRH targeted MTX–HSA NPs showed that increasing the amount of attached LHRH molecules on the surface of MTX–HSA NPs could increase the anti-tumor effect of LHRH targeted MTX–HSA NPs. As shown in Fig. 3, at day 21, the mean tumor volume in the LHRH 29.1–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 5.8–MTX–HSA NPs treated groups was 7.31 ± 2.93, 3.84 ± 3.03 and 3.14± 1.08-fold respectively lower than that of the non-targeted MTX–HSA NPs treated group (equivalent to 12.5 mg/kg free MTX).

As can be seen in Fig. 3, the LHRH targeting could decrease the size of tumors in LHRH targeted MTX–HSA NPs treated groups. By day 7, the average tumor volume in LHRH 29.1–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 5.8–MTX–HSA NPs treated groups (equivalent to 12.5 mg/kg MTX) were respectively 8.57 ± 4.45%, 27.26 ± 10.36% and 45.66 ± 16.77% of the initial tumor volume, whereas the average tumor volume in non-targeted MTX–HSA NPs treated mice (equivalent to 12.5 mg/kg free MTX) grew rapidly and reached 250.78 ± 29.01% of the initial tumor volume.

### 3.4. LHRH targeted nanoparticles prolonged survival of tumor-bearing mice

All mice were also followed to determine the length of survival. The survival rate of 4T1 tumor bearing mice is shown in Fig. 4. Both control group (saline) and free MTX treated group (12.5 mg/kg) exhibited rapid death. MST in control group and free MTX treated group (12.5 mg/kg) were 15.05 ± 0.79 and 17.5 ± 0.96 days respectively. The MST of mice treated with non-targeted MTX–HSA NPs (equivalent to 12.5 mg/kg free MTX) was 25.45 ± 0.23 days. Mice administered LHRH targeted MTX–HSA NPs showed significantly better survival rate compared to non-targeted MTX–HSA NPs, indicating the effectiveness of the LHRH targeted MTX–HSA NPs. The MST of LHRH 29.1–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 5.8–MTX–HSA NPs treated groups were 47.5 ± 0.43, 40.12 ± 0.12 and 32.5 ± 0.15 respectively. The ILS of 4T1 tumor bearing mice that treated with free MTX and non-targeted MTX–HSA NPs was found to be 16.66% and 66.66% respectively. Whereas ILS of mice that treated with LHRH 5.8–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 29.1–MTX–HSA NPs were 116.66%, 166.66% and 216.66% respectively. The MST and ILS of control and treated 4t1 tumor bearing mice are summarized in Table 2.

### 3.5. Evaluation of body weight loss of 4T1 tumor bearing mice

The animal weight of mice was recorded every 3 days. The results showed that there were no significant difference among the weight change of groups that treated with free MTX and non-targeted MTX–HSA NPs compared to the group that got only saline. Moreover, there were no significant difference among the weight change of the groups that treated with LHRH 5.8–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 29.1–MTX–HSA NPs (Fig. 5). But Fig. 5 shows that, there are significant differences between the weight changes of the groups treated with LHRH targeted MTX–HSA NPs and group that treated with non-targeted MTX–HSA NPs especially between day 9 and day 21 of treatment. The body weight loss lower than 8% of initial body weight is almost tolerable (Tseng et al., 2009). The body weight loss of tumor bearing mice

**Table 2**
The median survival time (MST) and increase in the life span (ILS%) of control, non-targeted and LHRH targeted MTX–HSA NPs[a] treated 4T1 tumor bearing mice.

| Group | Median survival time (day) | Life span increase (%) |
|---|---|---|
| Saline | 15.05 ± 0.79 | – |
| MTX | 17.51 ± 0.96 | 16.66 |
| MTX–HSA NPs | 25.45 ± 0.23 | 66.66 |
| LHRH 5.8–MTX–HSA NPs | 35.2 ± 0.15 | 116.66 |
| LHRH 17.3–MTX–HSA NPs | 40.12 ± 0.12 | 166.66 |
| LHRH 29.1–MTX–HSA NPs | 47.5 ± 0.43 | 216.66 |

[a] The dose was equivalent to 12.5 mg/kg of free MTX.

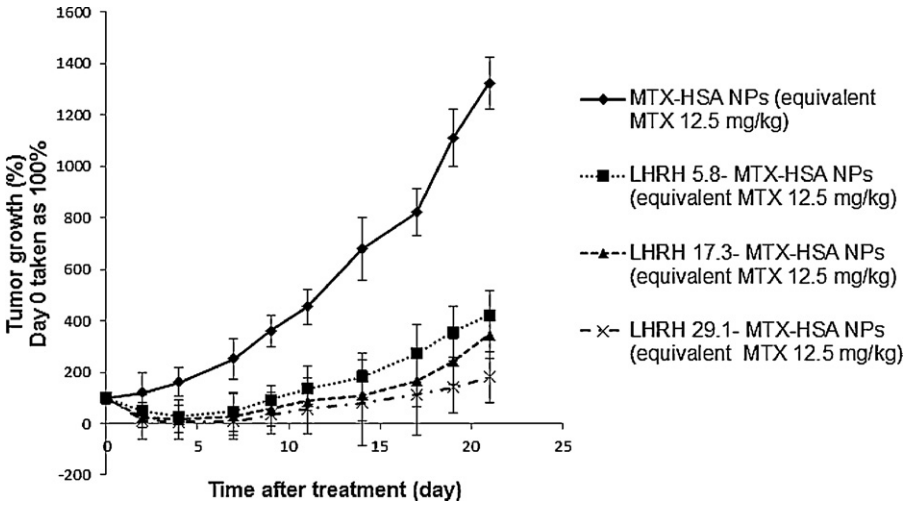


**Fig. 3.** Antitumor effect of MTX–HSA NPs, LHRH 5.8–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 29.1–MTX–HSA NPs on 4T1 tumor bearing mice. 4T1 tumor cells were implanted s.c. in Balb/c mice. The drugs were injected i.v. in a single dose (day 0). The doses were equivalent to 12.5 mg/kg of free MTX. Data are presented as mean ± SD of relative tumor volumes (day 0 taken as 100%).

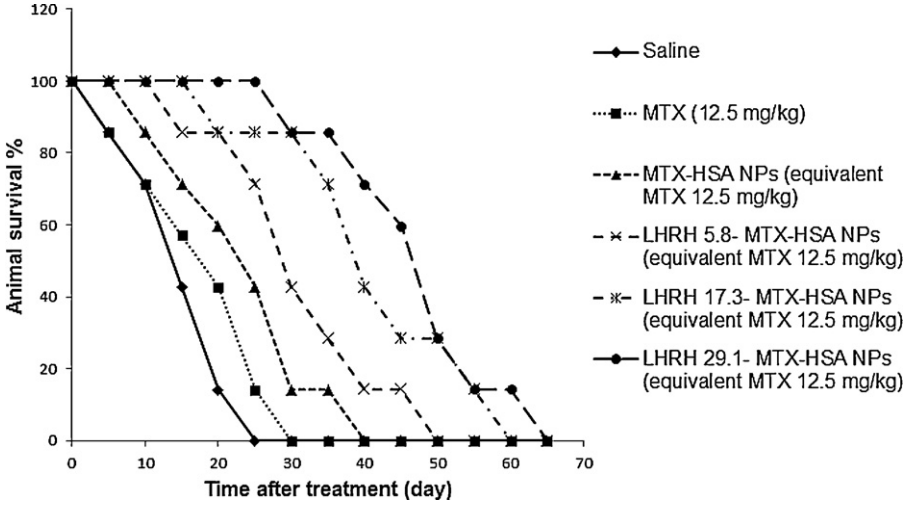


**Fig. 4.** Animal survival study. The 4T1 tumor bearing mice were treated with free MTX, MTX–HSA NPs, LHRH 5.8–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 29.1–MTX–HSA NPs. The drugs were injected i.v. in a single dose (day 0). The doses were equivalent to 12.5 mg/kg of free MTX. The curve reports the number of 4T1 tumor bearing mice still alive on different days.

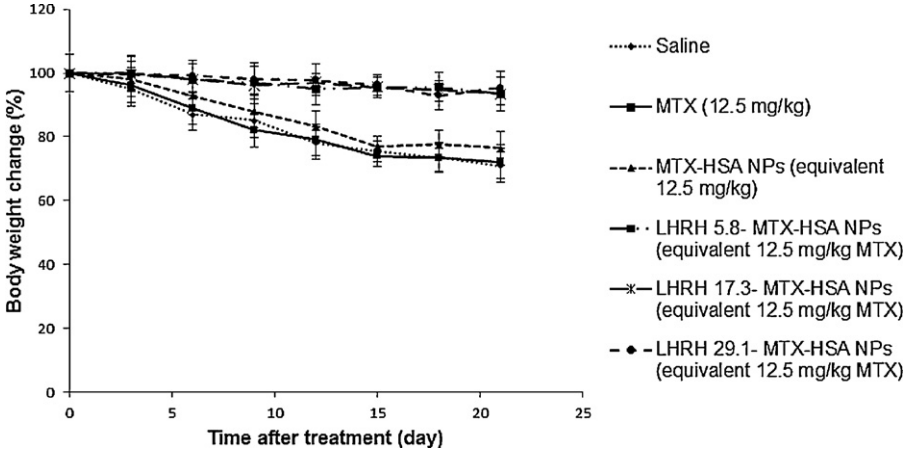


**Fig. 5.** Alteration of body weight of 4T1 tumor bearing mice treated with free MTX, MTX–HSA NPs, LHRH 5.8–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 29.1–MTX–HSA NPs. The drugs were injected i.v. in a single dose (day 0). The doses were equivalent to 12.5 mg/kg of free MTX.

that treated with LHRH targeted MTX–HSA NPs were lower than 8% of initial body weight after 21 days. However, mice that treated with free MTX and non-targeted MTX–HSA NPs lost 20.9 ± 4.5% and 17.3 ± 5.78% of their body weight after 12 days.

## 4. Discussion

Conjugation of cytotoxic drugs to HSA NPs could improve their antitumor efficacy and decrease their toxic side effects (Kratz, 2008). The free amino and carboxylic acid groups of HSA could be used for covalent coupling of cytotoxic drugs and targeting moieties (Zhang et al., 2004; Kreuter et al., 2007; Steinhauser et al., 2006; Esmaeili et al., 2009). Therefore HSA nanoparticles have been proposed as a suitable drug carrier system for targeted drug delivery to specific tumor sites (Kratz, 2008; Zhang et al., 2004). Several previous studies have demonstrated the expression of LHRH receptors in different cancer cells (Reubi, 2003; Schally and Nagy, 2003) and the use of LHRH functionalized nanoparticles for tumor targeting (Taratula et al., 2009; Minko et al., 2010). We therefore used LHRH as a model tumor targeting ligand in our study. LHRH was conjugated on the surface of MTX–HSA NPs using a carbodiimide reaction that we reported previously (Taheri et al., 2011b).

LHRH functionalizing of nanoparticles could significantly enhance the uptake of these nanoparticles in tumor cells. In our previous study, we used flow cytometry analysis and fluorescence microscopy for evaluation of the cellular uptake of LHRH targeted MTX–HSA NPs in tumor cells. The results showed that the uptake of LHRH targeted MTX–HSA NPs increased in the LHRH receptor positive T47D tumor cells compared to non-targeted MTX–HSA NPs. Moreover the uptake of LHRH targeted MTX–HSA nanoparticles did not increase in the LHRH receptor negative SKOV3 tumor cells, indicating the involvement of LHRH receptors in the cellular uptake of LHRH targeted MTX–HSA NPs. Based on the promising *in vitro* cytotoxicity and cellular uptake results, we investigated the anti-tumor efficacy of LHRH targeted MTX–HSA NPs in an *in vivo* model of 4T1 breast tumor. In this study, we firstly confirmed the expression of LHRH receptors on the 4T1 tumor cells by FITC labeled LHRH receptor antibody using fluorescence microscopy. T47D (LHRH receptor positive cells) and SKOV3 (LHRH receptor negative cells) cells were used as control positive and negative cells respectively. Fluorescence microscopy analysis of T47D, SKOV3 and 4T1 labeled cells with FITC labeled LHRH receptor antibody detected expression of LHRH receptors on the surface of 4T1 tumor cells similar to T47D (LHRH receptor positive cells). Breast tumor was inoculated in the female Balb/c mice by injection of 4T1 breast tumor cell solution in the breast region subcutaneously. *In vivo* tumor growth inhibition reinforced the results of the *in vitro* cytotoxicity studies. Free MTX by itself did not effectively inhibit the tumor growth *in vivo*. Non-targeted MTX–HSA NPs improved the anti-tumor effect of MTX on 4T1 breast tumors compared to free MTX. Similarly to *in vitro* study results, LHRH targeted MTX–HSA NPs showed the greatest tumor growth inhibition. This is probably due to the active targeting of LHRH targeted MTX–HSA NPs to the 4T1 breast tumors using LHRH molecules. The *in vivo* data showed that an i.v. injection of LHRH targeted MTX–HSA NPs even at a low dose of MTX (6.25 mg/kg) was effective at arresting the growth of 4T1 tumors in the tumor bearing mice. Not only was the mean tumor size maintained constant, but the tumor sizes in tumor bearing mice treated with LHRH 29.1–MTX–HSA NPs to less than 10% of their initial sizes 7 days after treatment. Tumor volumes at day 21 in mice treated with LHRH 29.1–MTX–HSA NPs, LHRH 17.3–MTX–HSA NPs and LHRH 5.8–MTX–HSA NPs was, respectively, 1143.15% ± 66.9%, 979.56% ± 53%, and 903.44% ± 28% less than that of those treated with non-targeted MTX–HSA NPs. Similar to *in vitro* results, LHRH targeted MTX–HSA NPs with more attached LHRH molecules on their surfaces showed stronger *in vivo* antitumor effects than LHRH targeted MTX–HSA NPs with less LHRH molecules. The average survival time of LHRH targeted MTX–HSA NPs treated groups was significantly extended compared with the non-targeted MTX–HSA NPs and free MTX treated groups. This may be attributed to the stronger anti-tumor activity of LHRH targeted MTX–HSA NPs compared to non-targeted MTX–HSA NPs and free MTX *in vivo*. The symptoms of body weight loss were observed in free MTX and non-MTX–HSA treated groups, but not in LHRH targeted MTX–HSA NPs treated groups, which might indicate that LHRH targeting could decrease the systematic toxicity (for example, disturbing food uptake) of MTX in LHRH targeted MTX–HSA NPs treated groups compared to free MTX and non-MTX–HSA treated groups. Consequently, LHRH targeting moieties could target MTX to the tumor site effectively and increase its antitumor effect sufficiently in tumor bearing mice.

## 5. Conclusion

The present study demonstrates that the LHRH targeted MTX–HSA NPs is effective for tumor treatment in a well-established animal model. Significant tumor growth delay were observed in 4T1 tumor bearing mice treated with LHRH targeted MTX–HSA NPs compared to non-targeted MTX–HSA NPs treated group, probably caused by active targeting of MTX–HSA NPs to the tumor site using LHRH molecules .The body weight loss of LHRH targeted nanoparticles treated groups was very low. The findings suggest that LHRH targeted MTX–HSA NPs may be useful for treatment of LHRH receptor positive cancer with an improved therapeutic index of MTX.

## References

Aslakson, C.J., Miller, F.R., 1992. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res. 152, 1399–1405.

David, A., Kopeckova, P., Minko, T., Rubinstein, A., Kopecek, J., 2004. Design of a multivalent galactoside ligand for selective targeting of HPMA copolymer–doxorubicin conjugates to human colon cancer cells. Eur. J. Cancer 40, 148–157.

Dharap, S.S., Wang, Y., Khandna, P., Qiu, B., Gunaseelan, S., Sinko, J.P., Stein, S., Farmanfarmaian, A., Minko, T., 2005. Tumor-specific targeting of an anticancer drug delivery system by LHRH peptide. PANS 102, 12962–12967.

Dinarvand, R., Sepehri, N., Manoochehri, S., Rouhani, H., Atyabi, F., 2011. Polylactide-co glycolide nanoparticles for controlled delivery of anticancer, agents. Int. J. Nanomed. 6, 877–895.

Esmaeili, F., Dinarvand, R., Ghahremani, M.H., Amini, M., Rouhani, H., Sepehri, N., Ostad, S.N., Atyabi, F., 2009. Docetaxel-albumin conjugates: preparation, in vitro evaluation and biodistribution studies. J. Pharm. Sci. 98, 2718–2730.

Esmaeili, F., Ghahremani, M.H., Ostad, S.N., Atyabi, F., Seyedabadi, M., Malekshahi, M.R., Amini, M., Dinarvand, R., 2008. Folate-receptor-targeted delivery of docetaxel nanoparticles prepared by PLGA-PEG-folate conjugate. J. Drug Target. 16, 415–423.

Geran, R.I., Greenberg, N.H., Mac Donald, M.M., Schumacher, A.M., Abbot, B.J., 1972. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother. Rep. 3, 1–103.

Günthert, A., Gründker, C., Bongertz, T., Schlott, T., Nagy, A., Schally, A., Emons, G., 2004. Internalisation of cytotoxic luteinizing hormone-releasing hormone analog AN-152 induces multi drug resistance 1(MDR-1)-independent apoptosis in human endometrial and ovarian cancer cell lines. Am. J. Obstet. Gynecol. 191, 1164–1172.

Gupta, U., Kumar Dhar Dwivedi, S., Kumar Bid, H., Konwar, R., Jain, N.K., 2010. Ligand anchored dendrimers based nanoconstructs for effective targeting to cancer cells. Int. J. Pharm. 393, 185–196.

He, Y., Zhang, L., Song, C., 2010. Luteinizing hormone-releasing hormone receptor-mediated delivery of mitoxantrone using LHRH analogs modified with PEGylated liposomes. Int. J. Nanomed. 5, 697–705.

Heppner, G.H., Miller, F.R., Shekhar, P.M., 2000. Nontransgenic models of breast cancer. Breast Cancer Res. 2, 331–334.

Jin, Y., Ren, X., Wang, W., Ke, L., Ning, E., Dua, L., Bradshaw, J., 2011. A 5-fluorouracil-loaded pH-responsive dendrimer nanocarrier for tumor targeting. Int. J. Pharm. 420, 378–384.

Kratz, F., 2008. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. J. Control. Release 132, 171–183.

Kreuter, J., Hekmatara, T., Dreis, S., Vogel, T., Gelperina, S., Langer, K., 2007. Covalent attachment of apolipoprotein A-I and apolipoprotein B-100 to albumin nanoparticles enables drug transport into the brain. J. Control. Release 118, 54–58.

Krishnaiah, Y.S., Satyanarayana, V., Dinesh Kumar, B., Karthikeyan, R.S., 2002. In vitro drug release studies on guar gum-based colon targeted oral drug delivery systems of 5-fluorouracil. Eur. J. Pharm. Sci. 16, 185–192.

Leuschner, C., Hansel, W., 2005. Targeting breast and prostate cancers through their hormone receptors. Biol. Reprod. 73, 255–260.

Lukyanov, A.N., Elbayoumi, T.A., Chakilam, A.R., Torchilin, V.P., 2004. Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anticancer antibody. J. Control. Release 100, 135–144.

Minko, T., 2004. Drug targeting to the colon with lectins and neoglycoconjugates. Adv. Drug Delivery Rev. 56, 491–509.

Minko, T., Patil, M.L., Zhang, M., Khandare, J.J., Saad, M., Chandna, P., Taratula, O., 2010. LHRH targeted nanoparticles for cancer therapeutics. Methods Mol. Biol. 624, 281–294.

Reubi, J.C., 2003. Peptide receptors as molecular targets for cancer diagnosis and therapy. Endocr. Rev. 24, 389–427.

Schally, A., Nagy, A., 2003. New approaches to treatment of various cancers based on cytotoxic analogs of LHRH, somatostatin and bombesin. Life Sci. 72, 2305–2320.

Steinhauser, I., Spankuchbm, B., Strebhardt, K., Langer, K., 2006. Trastuzumab modified nanoparticles: optimisation of preparation and uptake in cancer cells. Biomaterials 27, 4975–4983.

Taheri, A., Atyabi, F., Salman Nouri, F., Ahadi, F., Derakhshan, M.A., Amini, M., Ghahremani, M.H., Ostad, S.N., Mansoori, P., Dinarvand, R., 2011a. Nanoparticles of conjugated methotrexate–human serum albumin: preparation and cytotoxicity evaluations. J. Nanomater.

Taheri, A., Dinarvand, R., Atyabi, F., Ahadi, F., Salman Nouri, F., Ghahreman, M.H., Ostad, S.N., Taheri Borougeni, A., Mansoori, P., 2011b. Enhanced anti-tumoral activity of methotrexate–human serum albumin conjugated nanoparticles by targeting with luteinizing hormone-releasing hormone (LHRH) peptide. Int. J. Mol. Sci. 12, 4591–4608.

Tao, K., Alroy, J., Sahagian, G.G., 2008. Imagable 4T1 model for the study of late stage breast cancer. BMC Cancer 8, 228.

Taratula, O., Garbuzenko, O.B., Kirkpatrick, P., Pandya, I., Savla, R., Pozharov, V.P., He, H., Minko, T., 2009. Surface-engineered targeted PPI dendrimer for efficient intracellular and intratumoral siRNA delivery. J. Control. Release 140, 284–293.

Tseng, C., Su, W., Yen, K., Yang, K., Lin, F., 2009. The use of biotinylated- EGF-modified gelatin nanoparticle carrier to enhance cisplatin accumulation in cancerous lungs via inhalation. Biomaterials 30, 3476–3485.

Vredenburg, M.R., Ojima, I., Veith, J., Kee, K., Cabral, F., Sharma, A., Kanter, P., Greco, W.R., Bernacki, R.J., 2001. Effects of orally active taxanes on P-glycoprotein modulation and colon and breast carcinoma drug resistance. J. Natl. Cancer Inst. 93, 1234–1245.

Yang, W., Cheng, Y., Xu, T., Wang, X., Wen, L-p., 2009. Targeting cancer cells with biotin dendrimer conjugates. Eur. J. Med. Chem. 44, 862–868.

Zhang, L., Hou, S., Mao, S., Wei, D., Song, X., Lu, Y., 2004. Uptake of folate conjugated albumin nanoparticles to the SKOV3 cells. Int. J. Pharm. 287, 155–162.

Zhao, D., Zhao, X., Zu, Y., Li, J., Zhang, Y., Jiang, R., Zhang, Z., 2010. Preparation, characterization, and in vitro targeted delivery of folate-decorated paclitaxel-loaded bovine serum albumin nanoparticles. Int. J. Nanomed. 5, 669–677.