

RESEARCH PAPER

Docetaxel delivery using folate-targeted liposomes: in vitro and in vivo studies

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ABSTRACT

Objective(s): Folate-targeted liposomes have been well considered in folate receptor (FR) overexpressing cells including MCF-7 and 4T1 cells in vitro and in vivo. The objective of this study is to design an optimum folate targeted liposomal formulations which show the best liposome cell uptake to tumor cells.

Material and Methods: In this study, we prepared and characterized different targeted formulations and a nontargeted form as a control. Physicochemical analysis showed that the liposomes had homogeneous population and appropriate size to accumulate to tumor sites through the enhanced permeation and retention (EPR) mechanism. Moreover, we compared the cell uptake of folate targeted liposomal docetaxel compared to nontargeted liposomes in vitro.

Results: The in vitro drug release profile of the formulations at different time points showed none of the formulations did not has burst release. However, targeted liposomes accumulated in tumor tissue in vivo less than nontargeted formulations which could be attributed to their uptake by RES due to relatively greater size of targeted formulations. It is presumable that analyze the biodistribution process at longer time points and the molecular mechanisms behind the tissue accumulation could clear the issue.

Conclusion: We conclude that success in vitro studies holds the promise of folate targeting strategy and in vivo study merits further investigations.

Key words: Docetaxel Encapsulation, Folate Targeting, Liposomes, Tumor Drug Delivery

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INTRODUCTION

Currently, chemotherapy has been reported as one of the most commonly treatments for many types of tumors [1-4]. Several strategies are currently under evaluation to reduce dose-limiting toxicity and bypass the efflux pump of P-glycoprotein (P-gp), thereby improve the therapeutic efficacy of potent anticancer drugs. An often proposed strategy for targeting delivery of chemotherapeutics to cancer cells is the folate receptor (FR) [5]. FR is a 38 kDa glycosyl phosphatidylinositol membrane linked

glycoprotein that is overexpressed (100–300 times higher) at the surface of many types of human cancers, compared to low levels observed in most normal epithelial tissues [6]. Folic acid (FA) is a small molecular ligand which has high affinity ($K_d = 0.1-1 \text{ Nm/L}$) for the FR [7]. Folate has received great attention for its advantages including small size (M_r 441.4), stability during storage, low immunogenicity, ease of modification, low cost, and ready availability [8]. Folate-targeting strategy enables the liposomes to successfully deliver therapeutics agents; ranging from small imaging payloads up to DNA-loading formulations into FR overexpressing tumor cells [9-11]. Also, it

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can increase the cellular uptake and cytotoxicity compared to non-targeted liposomes and free forms of chemotherapeutics *in vitro* [12, 13]. These effects result from more efficient delivery of drugs after internalization into FR overexpressing tumor cells via receptor-mediated endocytosis [11, 14]. Despite many studies in tumor targeting strategies based on folate receptor, there are very few reports that describe *in vivo* fate of folate targeted formulations. The MCF-7 cell line, derived from metastatic site; breast, mammary gland, human, Homo sapiens, and 4T1 mammary cell lines, as murine mammary carcinoma cells clearly exhibit high expression of folate receptor. These cell lines have become proper models for investigating factors that affect FR-targeted delivery in an animal. Moreover, NIH/3T3 cell lines are standard fibroblast cell lines which have normal level of folate receptor expression [15].

Here, we prepared various docetaxel loaded nanoliposomes, determined the IC_{50} values of formulations on MCF-7 and NIH/3T3 cell lines and then investigated their binding to MCF-7 cells *in vitro*. Afterward, the biodistribution of the formulations were evaluated in BALB/c mice bearing 4T1 breast carcinoma tumors using ^{99m}Tc HMPAO method.

MATERIALS AND METHODS

Taxotere[®] vial was provided from Sanofi Aventis Company (France). Folate-poly(ethylene glycol)-distearoylphosphatidyl ethanolamine (FA-PEG 3350 -DSPE), 1,2-distearoylsn- glycerol-3 phosphoethanolamine-N-[methoxy (polyethylene glycol)- 2000] (mPEG3350-DSPE), hydrogenated soy phosphatidylcholine (HSPC), were prepared from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol.), RPMI 1640 (Roswell Park Memorial Institute medium), DMEM (Dulbecco's Modified Eagle's medium) were purchased from Sigma- Aldrich. [3-(4, 5-dimethylthiazol-2-yl)-2], diphenyltetrazolium bromide (MTT) was provided by Promega (Madison, WI). Fetal Calf Serum (FCS) and trypsin were purchased from Gibco (UK). Penicillin-streptomycin solution was from Invitrogen. ^{99m}Tc sodium pertechnetate was obtained by Kimia pakhsh ^{99m}Tc generator (Iran). Kit for the preparation of Hexamethyl propylene amine oxime (HMPAO) was according to the similar procedures of commercial kits with the formulation of CERETEC; 0.5 HMPAO and 7.6 μ g SnCl₂. All other chemical solvents and reagents were chemical grade.

Liposomes preparation

Liposomal formulations were prepared using the thin film hydration and extrusion methods, as previously described [16]. Briefly, docetaxel (at lipid-to-drug molar ratio of 20:1), the stock chloroform solution of mPEG2000-DSPE, cholesterol, HSPC, and FA-PEG 3350-DSPE at molar ratio of 0.00, 0.05, 0.1, and 0.2% were added. Then, rotary evaporator (Heidolph, Germany) and freeze-drier (VD-800F, Taitech, Japan) were used to remove the solvents from the lipid solutions. The Lipid film was then hydrated with histidine buffer (250 mM) at pH 7.00 and vortexed to completely disperse the lipids into the buffer. The resulting multilamellar vesicles were extruded through 200, 100, and 50 nm polycarbonate membranes to form unilamellar vesicles with uniform sizes. To purify the liposomal product, a purification method based on dialysis was used. In this method, free, not encapsulated histidine was separated using a 12-14-kDa molecular weight cut-off (MWCO) dialysis membrane (Spectrum, Houston, TX, USA) against sucrose/HEPES buffer (sucrose: 10%, HEPES: 10 mM, pH 7.4) for 24 hrs at room temperature. Final product was filtered through 0.45 mm syringe filter to remove possible crystal structures of DTX.

Size, zeta potential, drug content, and drug encapsulation Efficiency

Dynamic Light Scattering (DLS) instrument (Nano-ZS; Malvern, UK) was used to analysis size, polydispersity index (PDI), and zeta potential of the liposomes. The amount of DTX in the liposomes was measured by the high performance liquid chromatography (HPLC) according to US pharmacopeia instructions. KNAUER smart line HPLC (Berlin, Germany) was equipped with a Waters C18, 3.5 μ m, 150 \times 4.6 mm, 100Å column. The encapsulation efficiency (EE) of the liposomes was determined as drug concentration after dialysis/ initial amount of the drug used in the liposomes \times 100.

In vitro drug release

The *in vitro* drug release profile of the liposomal formulations was performed based on dialysis method as mentioned above. For this, 1 mL of liposomal formulations was immersed as packed sample in the dialysis bag (cut off 12 kDa) against 100 mL of HEPES - buffered sucrose as a release medium under magnetic stirring at 37 °C. 1 mL release media sucked out at 0, 1, 2, 4, 6, 12,

24, 48, and 72 hrs intervals, and then immediately replaced with an equal volume of the fresh medium.

The concentrations of drug in the samples were measured by HPLC. Finally, by multiplying the dilution factor, the real amount of DTX released from closed bags was determined. The error bars were presented with measurements performed at least in triplicates.

***In vitro* cellular uptake by flow cytometry**

This study was performed to compare the targeted and non-targeted liposomal formulation uptake by MCF-7 cells. For this, the liposomes labeled with fluorescent dye (Dil) were prepared using lipid film hydration method according to the above procedure.

Then, the cells at a density of 10^6 cells/ml were incubated with Dil-labeled liposomes (100 nmol/ml) at 37 and 4°C for 5 hrs. Untreated cells were used as control groups. Liposomes uptake by MCF-7 cells were analyzed by flow cytometry on the FL2 channel and the mean fluorescence intensity was measured.

Cytotoxicity study

MCF-7, a cell line overexpressing the folate receptor, and NIH/3T3 cells as a control group which has a normal expression of folate receptor were cultured in completed Dulbecco Modified Eagle Medium (DMEM) medium supplemented with 10% (v/v) Fetal Calf Serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were incubated in humidified 37°C incubator with 5% CO₂. Then, the cells were collected by trypsinization and seeded with density of 5000 cells/well and volume of 100 µL in 96-well plate.

After the cells prepared confluence, the FA-targeted and non-targeted nanoliposomes were dispersed in the medium at concentration of 500, 250, 120, 62.50, 31.25, and 15.62 µg/ml and incubated at 37°C for 24, 48, and 72 h. finally, the viability of cells was assessed using a MTT test and then IC₅₀ of formulations was calculated by CalcuSyn version 2.0 (CalcuSyn Software, USA). IC₅₀ is the drug concentration needed for 50% cell growth in a designated time period of the cell culture [17].

Animal study

Female BALB/c mice, 4–6 weeks old, were purchased from the Pasteur Institute (Tehran,

Iran). The mice received humane care according to the Ethical Committee and Research Advisory Committee of Tehran University of Medical Sciences under the protocol number of 9221148001.

Biodistribution study

Liposome radio labeling

This experiment was performed using radioactive technetium according to the modified ^{99m}Tc-HMPAO method. Briefly, 1.5 mCi of freshly prepared ^{99m}Tc-HMPAO was added to 1 ml of glutathione contained liposomes (100 mM) and incubated for 30 min at room temperature.

The labeled liposomes were purified with Sephadex G-50 column (Sigma-Aldrich, St Louis, MO, USA) equilibrated with NaCl 0.9 %. Finally, serum stability of the ^{99m}Tc-labeled liposomes was determined by instant thin layer chromatography-silica gel (ITLC-SG) at different time points [18].

Biodistribution study in animal

On day 0, 60 µl PBS buffer containing 5×10^5 4T1 breast carcinoma cells were inoculated subcutaneously in the right flank of each BALB/c mouse.

After two weeks when the wide tumors was approximately 5 mm, the mice were randomized into 4 groups of three mice each: 1) group of mice treated with liposomes containing 0.05 % FA, 2) group of mice treated with liposomes containing 0.1 % FA, 3) group of mice treated with liposomes containing 0.2 % FA, 4) group of mice treated with non-targeted liposomes. Then, radiolabeled liposomes (60 µCi per mouse) were intravenously injected through the lateral tail vein of each mouse. Finally, the mice of each group were euthanized for tissue collection at 12 hrs after liposomes administration. The organs of interest were removed, washed, weighted and the radioactivity of each organ was read by a gamma counter (Delshid, Tehran, Iran).

The results are presented in three different ways: (1) Mean percentage of injected dose per gram of organ (%ID/g), (2) Tissue/ Blood ratios, and (3) Tumor/Normal Tissue (T/NT) ratio.

Statistical analysis

GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for statistical analyses. The average of data is shown as the mean ± SD (standard deviation). P < 0.05 was considered for the significance of difference among groups.

Table. Characteristics and encapsulation efficiencies of encapsulated DTX liposomal formulations

Formulations- Molar ratio	Size				PDI ^a	Zeta potential (mV)	EE ^b (%)
	Z Average (nm)	Intensity (nm)	Volume (nm)	Number (nm)			
HSPC: mPEG2000-DSPE: DSPE- PEG(3350)-FA: Chol, (8.5/0.45/0.00/1) - 100 mM	109	128	100.2	61.75	0.16	-11	53
HSPC: mPEG2000-DSPE: DSPE- PEG(3350)-FA: Chol, (8.5/0.45/0.05/1) - 100 mM	126.9	148	119.2	91.75	0.18	-12.6	59
HSPC: mPEG2000-DSPE: DSPE- PEG(3350)-FA: Chol, (8.5/0.45/0.1/1) - 100 mM	129.9	157	117.7	65.6	0.20	-11.8	55
HSPC: mPEG2000-DSPE: DSPE- PEG(3350)-FA: Chol, (8.5/0.45/0.2/1) - 100 mM	146.4	177	148	80	0.11	-10.3	54

RESULTS

Physicochemical characterization of liposomes

Different folate-targeted liposomes were formulated at three different mole ratios of FA-PEG 3350-DSPE (0.05, 0.1, or 0.2). Physicochemical characteristics (size, PDI, zeta potential, and percent of encapsulation efficiency) of DTX loaded liposomes were summarized in Table 1. The average diameters of the nanoliposomes were approximately 127, 129, and 147 nm which show acceptable sizes for accumulation in the tumor based on EPR effect. Also, polydispersity indexes (PDI) less than 0.2 confirm homogeneity in their population. Encapsulation efficacy of formulations was approximately ~57 %.

In vitro drug release

The *in vitro* drug release profiles of the formulations at different time points were shown in Fig1.

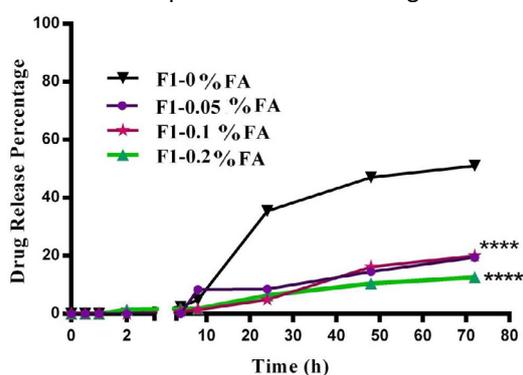


Fig.1. In vitro drug release profile of the folate targeted nanoliposomes compare to nontargeted counterparts. Values were presented from triplicate measurements for each formulation. At the time point of 72 hrs, all three targeted formulations exhibited significantly slower release than nontargeted form ($P < 0.0001$)

Based on these results, none of the formulations did not show burst release. However, all three targeted formulations exhibited significantly slower release than nontargeted form at the final time point i.e. 72 hrs ($P < 0.0001$). The effects of folate targeting on the DTX release from the nanoliposomes is thus clear, and folate incorporating can decrease and delay the drug release from the formulations.

Effect of ligand density on folate targeting to MCF-7 cells in vitro

We evaluated the cellular uptake of non-targeted or targeted formulations with various mole ratios of FA-PEG3350-DSPE by flow cytometry to determine the optimum ligand concentration for folate targeting to MCF-7 cells. The association of folate targeted liposomes with cells was dependent on ligand concentration.

The liposomes with 0.2 mol ratio of FA-PEG 3350 -DSPE had the greatest mean fluorescence (133 and 3.3 fold above unstained samples and non-targeted liposomes, respectively) compared to the other formulations at 37 °C. In addition, liposomes with 0.05 % FA-PEG3350-DSPE had a similar cellular association as formulations containing 0.1 % ligand (Fig 2).

According to our calculation, these results showed that 0.2 mol ratio of folate on the surface of the formulations was optimal for cell binding and could result in the most effective drug delivery to tumor cells.

Cytotoxicity study

Fig 3 shows cytotoxicity of DTX formulated in Taxotere® and nanoliposomes on NIH/3T3 and

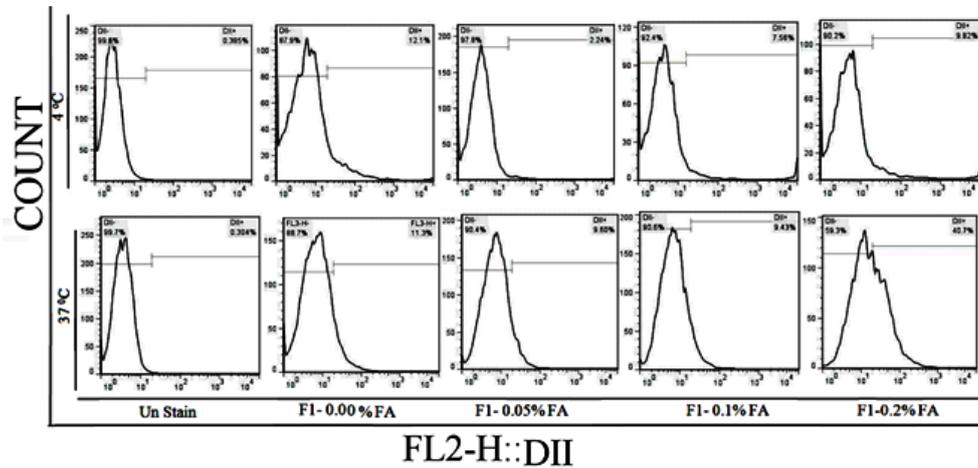


Fig 2. Cell uptake of targeted liposomes compare to non targeted formulations. MCF-7 Cells were incubated with liposomes labeled with Dil and containing FA-PEG 3350 -DSPE at 0.05, 0.1, or 0.2 mol ratios (mol %) and analyzed by flow cytometry. Upper panel: 5 h incubation at 4°C. Lower panel: 5h incubation at 37°C

MCF-7 cells at the same DTX dose at different time points. Two conclusions could be made from this table: 1) after 24 hrs cell incubation, cytotoxicity of Taxotere® formulation was significantly higher than different liposomal formulations. However, after 72 hrs cell incubation, mortality effect of Taxotere® formulation was comparable to that of the nanoliposomes.2) Non targeted liposomes showed comparable cytotoxicity with Taxotere® after 48 hrs time, however targeted formulations had significantly different effect at this time point. These conclusions could be contributed to the lower drug release manner of the nanoliposome formulations compare to Taxotere®.

They showed that the slower release delays the cytotoxic effect.

Animal study: biodistribution analysis

The Biodistribution of technetium-99m radiolabeled liposomes was considered in BALB/c mice 4T1 breast carcinoma tumor model at 12 hrs after i.v injection. The analyses data is presented separately for blood, liver, spleen, and tumor. As shown in Fig 4 A, non-targeted liposomes indicated significant prolonged circulation time compared to non-targeted forms (P<0.05). It could be attributed to the increased capture of targeted liposomes by phagocytic cells of the reticulo-endothelial system in spleen and liver as a result of their size and folate ligands (Fig 4 B). These results could be confirmed by Fig.4.B in which formulation with 2% ligand has demonstrated the significant higher accumulation in liver and spleen tissues.

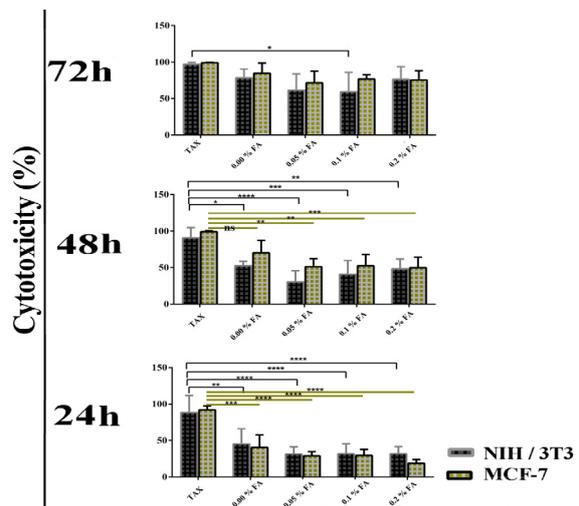


Fig.3. Cytotoxicity of DTX formulated in Taxotere® and targeted with 0.05, 0.01, and 0.2 % FA-PEG 3350 -DSPE (0.05 %FA, 0.1 %FA, and 0.2 %FA, respectively) and nontargeted nanoliposomes (0.00% FA) on NIH/3T3 and MCF-7 cells after 24, 48 and 72 hrs cell culture at the same DTX dose (n=6, mean ±SD). Statistically significant differences represented as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, ****P < 0.000.1

Moreover, Fig 4 C. showed that the targeted liposomes concentrations in tumor were higher than that of non-targeted liposomes at 12 hrs after i.v injection.

Also, the tumor concentrations of all three type of folate targeted liposomes were comparable without any significant difference (P>0.05).

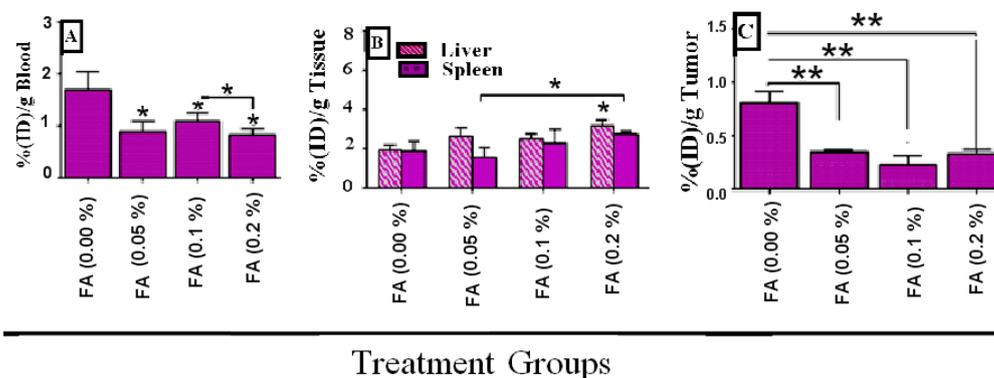


Fig 4. Biodistribution of nontargeted and targeted nanoliposomes after 12 hrs in blood (A), liver and spleen (B), and tumor (C) in BALB/c mice bearing 4T1 tumor 12 hrs after an i.v. administration of radiolabeled liposomes. Data are presented as mean percentage of injected dose per tissue (%ID/g) \pm SD (n = 3). Statistically significant differences represented as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

DISCUSSION

In the present study, non-targeted liposomes and liposomes containing folate were prepared and characterized. Then their biodistribution were investigated in mice bearing 4T1 breast carcinoma tumors. The size analysis showed that the average hydrodynamic particle sizes and polydispersity indexes were less than 200 nm and 0.2, respectively. This results mean that the liposomes have appropriate size and homogeneous population to accumulate to tumor sites through the EPR effect [19, 20]. Also, insertion of folate in to liposomes was caused to be increased the liposomes size and changed zeta potential. This observation could be a sign for conjugation of the folate moiety to the surface of liposomes [5, 21]. Wang et al., reported for the first time two drug delivery systems based on docetaxel-lipid-based-nanosuspensions to target folate receptor [22]. These folate-modified nanosuspensions were made up with FA-PEG 2000 –DSPE 0.5 mol% and have sizes greater than 200 nm. However, a direct correlation exists between particle size and uptake by the spleen [23]. Although due to the difference between nanoliposome with nanosuspension, we couldn't compare the results of this study with the results of our research; our study shows that an optimization in folate-ligand density could control the sizes of nanoparticles. Also, drug release of liposomes in our study was lower than that of nanosuspensions at similar time points indicating liposomal formulations could competitively be more appropriate for docetaxel delivery to tumor.

targeted liposomes showed lower release

which could be attributed to large size of targeted liposomes. This result is in line with the results of a number of previous findings which proved role of size in nanoparticle stability [24, 25]. We found that including 0.2 mol ratio of the folate ligand in the liposome formulations resulted in the best liposome cell uptake to MCF-7 cells *in vitro* (Fig 2). There is a possibility that these liposomes are able to efficiently interact with cells than other liposomes and prepared optimal accessibility of the folate moiety. However, formulations decorated with folate had significant lower concentration in the tumor. It could be attributed to their uptake by RES due to size. It is presumable that analyze the biodistribution process at longer time points and the molecular mechanisms behind the tissue accumulation could clear the issue. We conclude that success *in vitro* studies holds the promise of folate targeting strategy and *in vivo* study merits further investigations.

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