In conventional cancer chemotherapy, it is often necessary to increase the amount of cytotoxic drug administered in an exponential fashion in order to obtain a linear increase in killing of cancer cells. Thus, normal cells such as hair, skin, and gut cells may be affected by undesirable increase in non-specific cytotoxicity (1). Macromolecules or polymer conjugates offer the possibility to overcome several common drawbacks of low molecular weight (MW) drugs, such as high toxicity, poor selectivity, inappropriate pharmacokinetic profile, and low solubility. Several studies have demonstrated that the macromolecules or polymer conjugation can (i) increase drug half-life by reducing its kidney clearance (2,3), (ii) change distribution and pharmacokinetic of drugs (4–7), and (iii) enhance water solubility even to highly hydrophobic drugs (8,9). Furthermore, high molecular weight conjugates can selectively accumulate into tumors by a passive targeting mechanism known as enhanced permeability and retention (EPR) effect, which is not achievable by low MW drugs (10–12).

Enhanced permeation and retention (EPR) effect essentially relies on the pathophysiological properties of the target tissue. Nanomedicines with improved circulation time exploit the pathophysiological fact that solid tumors tend to present with a tortuous and poorly differentiated vasculature, which are immature, highly irregular, and abnormal with the size of fenestrae range from a few to hundreds of nanometers, that in contrast to the vasculature in healthy tissues, allows for the extravasation of carrier materials with sizes of up to several hundreds of nanometers. Due to lack of functional lymphatics in solid tumors, tumor tissues are unable to eliminate extravazated nanomaterials. Thus, long-circulating nanomedicines accumulate in tumors over time (7,13,14).

EPR or passive targeting alone might not be sufficient to reach a successful cancer treatment. Therefore, active
cancer cell targeting or ligand-mediated targeting is employed (15). In this method, specific ligands are conjugated to the macromolecular or polymeric backbone. These targeting residues are usually peptides, hormones, vitamins, or growth factors that are recognized by receptors overexpressed in tumor cells (16–18). Long circulation times will allow for effective transport of nanoparticles to the tumor site through both the EPR effect and active targeting of molecule(s), increasing the chance of endocytosis of the nanoparticles (16,19). Small, poorly immunogenic, tumor-specific targeting molecules such as vitamins and small molecules as nutrients are used frequently for this purpose, because rapidly dividing cells such as cancer cells require more nutrients for their survival and as a consequence they overexpress receptors to uptake enough nutrients (1). Considerable evidence is now accumulated which suggests that both biotin and folic acid have potential use in targeted delivery of imaging agents and pharmaceuticals to tumor cells (20–27).

Folic acid or folate is a vitamin required for one-carbon transfer reactions in several metabolic pathways and is essential for the biosynthesis of nucleotide bases and consumed in elevated quantities by proliferating cells (6). Folates are oxidized form of folic acid, or physiologically and biosynthetically active reduced tetrahydrofolate forms. Cellular uptake of folates proceeds through three main routes: (i) the proton-coupled folate transporter (PCFT/SLC46A1), which is responsible for intestinal folate absorption at the acidic pH of the upper small intestine, (ii) the ubiquitously expressed reduced folate carrier (RFC/SLC19A1) with very low affinity for folic acid, which is the primary pathway for reduced folate uptake into various tissues under physiological pH, and (iii) folate receptors (FRs) which are uptake folates via endocytosis, as folates cannot directly penetrate the cell membrane because of their hydrophobic anionic nature (28). FRs are glycosylphosphatidylinositol-anchored proteins that bind folic acid and 5-methyltetrahydrofolate with high affinity ($K_a = 10^{9–10^{-10}} \text{mol}^{-1}$). Of the four known isoforms ($\alpha$, $\beta$, $\gamma$, and $\delta$), FR-$\alpha$ and FR-$\beta$ are anchored to the plasma membrane and bind folic acid with the highest affinity (28,29). Cells that express FR-$\alpha$ are more efficient in folate uptake because FR-$\alpha$ binds folic acid with high binding affinity. In normal tissues and organs, FR-$\alpha$ expression is restricted to only a few sites, which include kidney, lung, choroid plexus, and placenta, where FR-$\alpha$ is confined to the luminal surface of polarized epithelia and, therefore, is not in contact with circulating folates or intravenously administered folic acid conjugates (28,30). FR-$\beta$ expression is restricted mainly to the placenta and white blood cells of myeloid lineage, including activated macrophages (28). Numerous studies have shown that FRs are markedly overexpressed on the surface of various tumor types, including ovarian, kidney, lung, brain, endometrial, colorectal, pancreatic, gastric, prostate, testicular, bladder, head and neck, and breast cancers, as well as non-small cell lung cancer (31–33). Evidence also suggests that FR expression increases with advancing disease stage and that overexpression of FR-$\alpha$ is a negative prognostic factor for breast, colorectal, ovarian, and endometrial cancer (27,28). Thus, FR is viewed as a therapeutic target that may provide an effective option for targeted personalized cancer therapy (28). Several researches showed that folate-conjugated macromolecular carriers could increase the uptake of anticancer drugs in tumor cells (6,21,28,33–35). A conjugation system for the delivery of vinblasticine targeted with folic acid is in phase III clinical trial (36,37).

Biotin (vitamin $B_7$, vitamin $H$) belongs to a category of essential micronutrients required for normal cellular functions (38). Rapid proliferations of cancer cells require extra biotin. Consequently, the receptors involved in the uptake of biotin are overexpressed in many cancer cells, such as leukemia (L1210FR), mastocytoma (P815), ovarian (OV 2008, ID8), colon (Colo-26), lung (M109), renal (RENCA, RD0995), and breast (4T1, JC, MMT06056) cancer cell lines (39). Recently, several researches showed that biotin-conjugated macromolecular carriers were able to increase the uptake of anticancer drugs in tumor cells (40–45). Thus, the specific interactions between biotin and its receptors may be exploited for targeted drug delivery (23,46–48).

HSA is emerging as a versatile protein carrier for drug targeting and albumin-based nanoparticle carrier systems represent an attractive strategy, because a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule (49). It is the most abundant plasma protein (35–50 g/L human serum) which consists of 585 amino acids, with a molecular weight of 66.5 kDa, synthesized in the liver at a rate of 10–15 g daily, and exhibits an average half-life of 19 days. HSA is an acidic, very soluble protein that is extremely robust. It is stable in the pH range of 4–9, soluble in 40% ethanol, and can be heated at 60 °C for up to 10 h without deleterious effects (5). These properties as well as its preferential uptake in tumor and inflamed tissues, ready availability, biodegradability, and lack of toxicity and immunogenicity make it an ideal candidate for drug delivery (5,50,51). Two principle albumin-based technologies have been developed in the past 15–20 years. In one approach, lipophilic drugs and HSA are passed under high pressure through a jet to form albumin-drug nanoparticles (it has no chemical bond, drugs were physically loaded), and in second approach, hydrophobic drugs are covalently conjugated to HSA to provide for self-aggregation of the conjugated macromolecules (50). Abraxane®, one of the commercialized targeted drug delivery systems, is an albumin-bound form of paclitaxel using the proprietary technology developed by Abraxis Biosciences (52). It is a water soluble galenic formulation that comprises albumin/paclitaxel nanoparticles with a diameter of $\sim$130 nm which is avoiding the use of cremophor EL. Abraxane® is the first albumin-based drug delivery system approved by the US-FDA for the treatment

Docetaxel (DTX) obtained from chemical modification of the 10-deacetyl-baccatin III extracted from the needles of Taxus baccata (European yew) blocks G2/M phase of cell cycle and prevents microtubules depolymerization (53,54). Because of low solubility of DTX, polysorbate 80 and ethanol are used in its conventional formulation commercialized as Taxotere® by Sanofi. The use of polysorbate 80 may alter the pharmacokinetics of DTX, which subsequently may induce variability of the activity and toxicity profiles, hypersensitivity, peripheral neuropathy, neutropenia, and stomatitis/mucositis, and other common interactions (4,53,55).

The aim of this study was to use the advantage of both EPR effect and active targeting of nanoparticles using targeting moieties to increase the specific uptake of the drug delivery system by cancer cells. These drug delivery systems (DDS) are targeted by dual methods. At first, DDS is accumulated in tumor interstitium which can cause increase in the concentration of DDS. Because of acidic pH of interstitium, some of DTX may release there. The targeting moiety (FA or biotin) will attach to their receptors and cause internalization of DDS. After internalization, HSA was used as cell nutrition and remaining DTX was released in tumor cells.

Methods and Materials

Materials

HSA as lyophilized powder, HABA/Avidin reagent kit, 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC), N, N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and triethylamine (TEA) were purchased from Sigma-Aldrich (Seelze, Germany). Anhydrous DTX was purchased from Xingcheng Chemphar Co. (Zhejiang, China). Sulfo-N-hydroxysuccinimide (Sulfo-NHS), succinic anhydride, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye were purchased from Merck (Darmstadt, Germany). Folic acid and biotin supplied from DSM (Heerlen, the Netherlands) and kindly are donated by Hakim Pharmaceutical Co. (Tehran, Iran). Bromocresol green reagent was supplied by Pars Azmun Co. (Karaj, Iran). Dialysis membrane with molecular weight cutoff of 12 KD was purchased from Spectrum Labs (California, USA). Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, RPMI 1640, fetal bovine serum (FBS), tripsin, penicillin, and streptomycin were purchased from BioSera (Boussens, France). Ultra-purified water was used throughout the analysis, and all other chemicals were of analytical grade. MDA-MB-231, 4T1 and A549 were obtained from national cell bank of Iran (Pasteur Institute of Iran, Tehran, Iran). BALB/c mice (6–8 weeks, female, ~21 g) were provided by Animal Care Center, Pasteur Institute of Iran.

Methods

Synthesis of succinyl docetaxel

SUC-DTX was synthesized by previously reported method (56). As a representative example, 250 mg (0.3095 mmol) of DTX anhydrous and 62 mg (0.62 mmol) succinic anhydride was dissolved in 3 mL dichloromethane (DCM) which is dried just before by anhydrous sodium sulfate. Then, 108 μL TEA (2.5 molar excess of DTX) was added to this mixture and stirred for 48 h. For purification of SUC-DTX, product of reaction was mixed with 3–5 volumes fold of phosphate-buffered saline (PBS; 0.1 M, pH = 9.0) and stirred 3–4 h. Then, pH of the mixture reduced to 4.0 by orthophosphoric acid (H3PO4) 85% and stirred again for 3–4 h. Then, 10 mL of ethyl acetate was added to this mixture and stirred for 2 h to dissolve SUC-DTX. Organic phase was separated in separator funnel, and liquid–liquid separation steps were repeated triplicate to ensure complete SUC-DTX extraction. The organic phase washed twice by 10 mL deionized water (DW) to remove all of water soluble residues. Finally, the organic solvent was evaporated at reduced pressure to obtain a light tan precipitate. This precipitate was subjected to 1HNMR (AC 500 spectrophotometer; Bruker, Leipzig, Germany) and HPLC analysis to identify and check the purity of SUC-DTX.

Synthesis of docetaxel-HSA conjugates

SUC-DTX (104 mg, ~0.1 mmol) was activated by EDC (80 mg, 0.4 mmol) and NHS (44.0 mg, 0.38 mmol) in 1 mL mixture of dimethyl sulfoxide (DMSO); dimethyl formamide (DMF) (70:30) for 4 h. HSA (66.5 mg, 1 μmol) was dissolved in 10 mL PBS (0.1 M, NaCl 0.05 M, pH = 8). Activated SUC-DTX solution was added drop-wise to HSA solution and stirred overnight at room temperature. Product of the reaction was centrifuged in 2500 g for 5 min to remove insoluble by-products. Then, the supernatant was dialyzed (MW cutoff, 12 KD) against 1 L PBS (0.1 M, NaCl 0.05 M, pH = 7.4) twice to remove organic solvent and water soluble by-products. Dialyzed liquid was centrifuged again to remove unreacted DTX and other insoluble by-products which were precipitated after removing organic solvents. This solution was used for preparation of nanoparticles or lyophilized for further studies.

Synthesis of HSA–folic acid conjugate

FA (128 mg, 0.3 mmol) was dissolved in 1 mL DMSO. Then, DCC (240 mg, 1.2 mmol) and NHS (120 mg, 1.08 mmol) was added to above solution and stirred for 4 h to activate FA. HSA (66.5 mg, 1 μmol) was dissolved in 10 mL PBS (0.1 M, pH = 9.1). Activated FA was added drop-wise to HSA solution and stirred overnight. Product of the reaction was centrifuged in 2500 g for 5 min to remove insoluble by-products, and the supernatant was dialyzed (MW cutoff 12 KD) against 1 L PBS (0.01 M, pH = 8.5) twice to remove organic solvent, water soluble

by-products, and unreacted FA. Dialyzed liquid was centrifuged again. The supernatant was used for preparation of DTX-HSA+HSA-FA nanoparticles or lyophilized for further studies. All of processes with FA took place in dark condition to protect FA from light.

**Synthesis of docetaxel-HSA-folic acid conjugate**

To save NH₂ residues for DTX attachment, amount of FA was reduced compare to previous HSA-FA conjugate. FA (8 mg, 18 μmol) was activated by DCC (15 mg, 72 μmol) and NHS (7.5 mg, 65 μmol) and reacted with HSA (66.5 mg, 1 μmol) which was dissolved in 10 mL PBS (0.1 M, pH = 9.1) by above-mentioned method. The product was centrifuged in 2500 g for 5 min, and the supernatant was dialyzed (MW cutoff 12 KD) against 1 L PBS (0.01 M, pH = 8.5) and then dialyzed again against 500 mL PBS (0.1 M, NaCl 0.05 M, pH = 8) to remove remaining materials and to replace medium by suitable buffer for DTX attachment (PBS 0.1 M, NaCl 0.05 M, pH = 8). Dialyzed liquid was centrifuged again and subjected for further reactions.

To attach DTX to HSA-FA conjugate, SUC-DTX was reacted with HSA-FA conjugate by the same amounts and method which was used for synthesis if DTX-HSA conjugate. Product of reaction was centrifuged in 2500 g for 5 min, and the supernatant was dialyzed (MW cutoff 12 KD) against 1 L PBS (0.01 M, pH = 8.5) for 5 min to remove insoluble by-products, and the supernatant was dialyzed against 1 L PBS 0.01 M, pH = 8.5 (MW cutoff 12 KD) for twice to remove organic solvent, water soluble by-products, and unreacted FA. Dialyzed liquid was centrifuged again and subjected for further studies.

**Synthesis of HSA-Biotin conjugate**

To attach biotin to HSA, similar method as HSA-FA conjugation was used. Biotin (73.3 mg, 0.3 mmol) was activated by DCC (240 mg, 1.2 mmol) and NHS (120 mg, 1.08 mmol) in 6 mL DMF for 4 h. HSA (66.5 mg, 1 μmol) was dissolved in 10 mL PBS (0.1 M, pH = 9.1). Activated biotin solution was added drop-wise to HSA solution and stirred overnight. The product was centrifuged in 2500 g for 5 min to remove insoluble by-products, and the supernatant was dialyzed against 1 L PBS 0.01 M, pH = 8.5 (MW cutoff 12 KD) for twice to remove organic solvent, water soluble by-products, and unreacted biotin. Dialyzed liquid was centrifuged again. The final solution was used for preparation of DTX-HSA+HSA-biotin nanoparticles or lyophilized for further studies.

**Synthesis of DTX-HSA-Biotin conjugate**

HSA-biotin conjugate was prepared by the same amount as mentioned above. Product of reaction was centrifuged and the supernatant was dialyzed (MW cutoff 12 KD) against 1 L PBS (0.01 M, pH = 8.5). Then, it dialyzed again against 1 L PBS (0.1 M, NaCl 0.05 M, pH = 8) to remove remaining materials and replace medium by suitable buffer for DTX attachment. Dialyzed liquid was centrifuged again.

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**Figure 1:** Flowchart of synthesis of drug conjugates and preparation of nanoparticles (A) DTX-HSA-FA conjugate, (B) DTX-HSA-HSA-FA nanoparticle, (C) DTX-HSA-biotin conjugate, and (D) DTX-HSA-HSA-biotin nanoparticle preparation.
DTX was attached to HSA-biotin conjugate as same as it attached to HSA-FA conjugate (Figure 1B).

**Preparation of DTX-HSA+HSA-FA and DTX-HSA+HSA-Biotin nanoparticles**

To prepare DTX-HSA+HSA-FA nanoparticles, 10 mL of HSA-FA conjugate (FA/HSA molar ratio of 32) solution mixed with 30 mL of DTX-HSA conjugate solution and sonicated in bath sonicator for 2–3 min. Suitable amount of mixture of ethanol + acetone (50:50) was added to this mixture during 30 seconds under probe sonicator (Misonix S-4000, New York, USA) by amplitude of 30 in ice bath, and sonication was continued for 3 min. This mixture was homogenized by high pressure homogenizer (HPH 2000/4-S-4000, New York, USA) by amplitude of 30 in ice bath, and sonication was continued for 3 min. This mixture was 92%. Results are reported as % mean ± SE values collected from three measurements.

To prepare DTX-HSA+HSA-biotin nanoparticles, 25 mL of HSA-biotin conjugate (biotin/HSA ratio 8) solution mixed by 30 mL of DTX-HSA conjugate solution and further processes was carried out the same as DTX-HSA+HSA-FA nanoparticles method of preparation which mentioned above (Figure 1D).

DTX-HSA nanoparticles were also prepared as control for cell viability studies. 30 mL of DTX-HSA conjugate solution sonicated and homogenized by the same process which mentioned above.

** Succinyl docetaxel determination**

To determine yield of the reaction and remained by-product, HPLC analysis was conducted. The chromatographic analysis was performed on a Knauer HPLC system (Berlin, Germany) equipped with K-1001 Knauer WellChrom pump and S-2500 UV Knauer WellChrom detector. The elution condition was developed using a mixture of acetonitrile and water (65:35 v/v) with a flow rate of 1.0 mL/min on a C18 column (MZ-analytical column, PerfectSil Target ODS-3, 250 × 4.6 mm id, 5-μm particle size) by UV detection at 229 nm. Chromgate software (version: 3.1.7) used for data acquisition and analysis. Recovery of DTX in the liquid–liquid extraction method was 92%. Results are reported as % mean ± SE values collected from three measurements.

** Determination of DTX in conjugates and nanoparticles**

To prepare a sample for DTX assay in the conjugates and nanoparticles, 20 mg of lyophilized powder of each conjugate or nanoparticles was dispersed in 3-mL ethyl acetate and stirred for 3 h to dissolve all unbounded DTX. This mixture was centrifuged by 2500 g for 3 min. The supernatant was separated, its solvent was evaporated, and the remaining was dissolved in 1 mL methanol for further analysis by HPLC. The results show unbounded DTX.

Five milliliters of acetate buffer (0.1 M, pH = 3) was added to precipitate of centrifuge and stirred at least 5 h to allow cleavage of esteric bond of SUC-DTX. Three milliliters of ethyl acetate was added to this solution and stirred 2–3 h, and two phases was separated. This step repeated triPLICATE. All of the organic phase was collected and washed twice by 10 mL DW. The solvent of organic phase was evaporated, and the remaining was dissolved in 1 mL methanol for further analysis by HPLC. The result shows bound DTX. HPLC condition that used for DTX assay in conjugates and nanoparticles was as same as that mentioned in above except its mobile phase which was acetonitrile 60%: DW 40%.

**FA assay**

Calibration curve of FA was drawn in 353 nm (Optizen 3220 UV Spectrophotometer) in the range of 20–70 μg/mL using 0.08 μM HSA in DW solution as blank. To assay FA in conjugates or nanoparticles, suitable amount of them was dissolved in DW and their absorbance was determined in 353 nm. The amount of FA was determined using by calibration curve line equation.

**Biotin assay**

HABA/Avidin reagent kit was used to assay biotin. Based on the manufacturer’s instruction, 10 mL DW was added to the reagent. Then, 900 μL of orange color solution transferred to a 1-mL disposable cuvette and its absorbance read in 500 nm. One hundred microliters of biotin conjugates or nanoparticles samples was added to this cuvette and mixed gently by inversion and read A500. Amount of biotin was determined by following formula:

\[ \Delta A_{500} = 0.9 \ (A_{500} \text{ HABA/AVIDIN}) - A_{500} \text{ HABA/AVIDIN + sample} \]

(0.9 = Dilution factor of HABA/Avidin upon addition of sample)

\[ \mu \text{mol biotin/mL} = (\Delta A_{500}/34) \] (10).

**HSA assay**

HSA assay was carried out using bromocresol green reagent which purchased as kit containing 0.26 mM bromocresol green in citrate buffer (30 mM). In the presence of HSA, color of solution changed from dark green to greenish blue. Intensity of color is proportional to HSA concentration. To assay HSA, a calibration curve was drawn. For this purpose, 200 μL HSA solution in PBS (0.1 M) by concentration range 1–5 mg/mL was added to 2 mL of bromocresol green reagent, incubated in 37 °C for 10 min,
and its absorbance was read in 546 (Optizen 3220 UV Spectrophotometer) while PBS (0.1 n) used as blank.

To assay of HSA in conjugates or nanoparticles, their solution was added to reagent solution as above-mentioned manner and read at 546 nm, concentration of HSA was determined using calibration curve formula.

**In vitro cytotoxicity**

Cell lines maintained in DMEM supplemented with 1% L-glutamine, 10% FBS, penicillin (100 IU/mL), and streptomycin (100 IU/mL) in a humidified atmosphere of 5% CO₂ at 37 °C in an incubator. When the cell confluency of 70% was achieved, the cells were trypsinized and subcultured.

The cytotoxicity of the conjugates and nanoparticles was determined using a 96-well plate format in quadruplicate with increasing doses. Each well contained approximately 5000 cells in 100 µL of cell culture media and incubated at 37 °C. DTX-HSA+HSA-FA and DTX-HSA+HSA-biotin were added as stock solutions in the culture medium, while free DTX was added as a DMSO stock solution (the final concentration of DMSO in culture medium was 0.5%). Cell viability was measured after 72 h when the treatments were aspirated off and replaced with PBS-containing MTT (5 mg/mL). Cells were then incubated for an additional 4.0 h. The formed formazan crystals were then dissolved in 100 µL of DMSO, and the absorbance was measured using microplate reader (Anthos 2020, Austria) at 570 nm against 690 nm. Cell viability was calculated as below:

\[
\text{Cell viability} = \frac{\text{Optical density of treated cells}}{\text{Mean optical density of control}}
\]

Untreated cells were considered as control with 100% viability. The results were expressed as mean values ± SD of four measurements.

**In vivo studies**

The in vivo efficacy of DTX-HSA-FA and DTX-HSA-biotin conjugates was evaluated in vivo in 6- to 8-week-old female BALB/c mice with average body weight of 21 g. Mice were kept under specific pathogen-free (SPF) conditions with controlled temperature at 22 ± 1 °C. They were maintained in a 12-h light/dark schedule with ad libitum food and water except during experimental procedures. Animals were allowed 7 days to acclimatize to the laboratory environment including handling before the beginning of injections. All animal experiments were conducted in full compliance with guidelines approved by the Animal Care Committee of Tehran University of Medical Sciences. The protocol (No. 357) was approved by the Committee of Ethics of the Faculty of Sciences of Tehran University.

To prepare a xenograft model, 5 × 10⁶ 4T1 murine breast cancer cells per mouse were dispersed in 320 µL PBS-containing culture medium and injected subcutaneously under shoulder skin to induce breast cancer tumor. When the tumors were palpable (more than 100 mm³), the mice were randomly divided into four groups (4–6 mice/group) including control group, free DTX group, DTX-HSA-FA conjugate group, and DTX-HSA-biotin conjugate group. Taxotere® 10 mg/kg, or DTX-HSA-FA (equiv. 5 mg/kg DTX) or DTX-HSA-biotin (equiv. 5 mg/kg DTX) and PBS as control were injected weekly via tail vein.

To determine tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined by external caliper. Tumor volume and survival were monitored. Tumor volume based on caliper measurements was calculated by the following formula (57)

\[
\text{Tumor volume} = \frac{1}{2} (\text{length} \times \text{width}^2)
\]

**Results and Discussion**

**Synthesis of drug conjugates**

To prepare SUC-DTX, succinic anhydride in the presence of TEA was reacted with DTX anhydrous in DCM for 48 h and purified by mixing with PBS (reducing pH from 9.0 to 4.0) and extracted by ethyl acetate. DTX has only –OH residues as functional groups, thus a succinyl group attached to anhydrous DTX to produce a –COOH group on DTX which can activate and react with amine groups on HSA to make DTX-HSA conjugate. On the other hand, succinyl group attached to DTX by an acid labile esteric bond which can break down in acidic environment of tumor interstitium and release the drug from conjugate. The method of SUC-DTX synthesis was developed by inspiration of Lee et al. (58) method with major modification. The old method used pyridine as a catalyst for SUC-DTX synthesis, but removal of the residue of pyridine from the final reaction product is too difficult. Also, SUC-DTX was purified on a silicagel column which is very time-consuming, need large amounts of organic solvents, and yield of reaction was too low. In our method, TEA was used instead of pyridine, which is volatile and can be removed easily. SUC-DTX was purified by pH changing and was extracted by little amounts of organic solvents. For purification, at first, the product of reaction was dispersed in an alkaline buffer to ionize all succinates and make them soluble in water, a milky emulsion was achieved. Then, pH of mixture was reduced. It causes to deionization of succinates, including SUC-DTX. Succinic acid is soluble in water and remains in aqueous phase, but SUC-DTX was precipitated as light brown paste which is extracted by little amount of ethyl acetate. This product was compared with the product which was obtain from Lee et al. method (58) by thin-layer chromatography (TLC) and has the same retention time (Data not shown).

The product of SUC-DTX synthesis structure was compared with DTX by ¹H NMR (4) (Bruker AC 500 spectrophotometer, Germany) in CDCl₃ as shown in Figure 2. The
structure of SUC-DTX was confirmed by comparison of DTX and their succinate derivative. There is no peak at 2.6–3.0 ppm in the $^1$H NMR of DTX. The presence of multiple peaks at 2.6–3.0 was attributed to succinyl moiety in SUC-DTX. Other peaks from 1 to 2.5, 3.8 to 6.2, and 7.2 to 8.1 are related to the DTX backbone. In the other hand, the mass spectra of SUC-DTX were acquired from an Agilent mass spectrometer at 70ev (EI-positive mode). The molecular ion at m/z 908 did not observe. Instead of, several low intensity peaks at 799, 754, 710, and 669 were observed. The ion at 799 could be attributed to loss two carboxyl groups and H$_2$O. The formation ion at m/z 754 could be explained to the losing of a carboxyl group from ion at 799. The ions at 105 (PhCO), 77 (Ph), and 51 were observed with the relative intensity of 100, 45, and 18, respectively. All in all, the new method enables replacing pyridine with TEA, which could be removed more easily than pyridine, while it leads to a more reproducible and less sophisticated reaction condition and less subsequent workup.

To determine the yield of reaction and remaining of other by-products, SUC-DTX obtained from this method was subjected to HPLC analysis. The chromatogram showed that around 94% of reactant was changed to SUC-DTX, and remaining by-products were negligible (Data not shown).

For synthesis of DTX-HSA conjugate, SUC-DTX was activated by EDC and NHS in DMSO: DMF and reacted with HSA in PBS (0.1 M, NaCl 0.05 M, pH = 8). It is purified by dialysis against PBS (0.01 M pH = 7.4). The DTX content was determined using HPLC method. The synthesis method of DTX-HSA conjugates was developed based on Esmaeili et al. (4) method with some modifications. Esmaeili et al. used sulfo-NHS to attach SUC-DTX to HSA. Also, the molar ratio of SUC-DTX to HSA was 30–40, which resulted in 1.3% drug loading (a DTX/HSA ratio of around 1.0). To modify this method, sulfo-NHS was replaced by NHS which is very cheaper. The molar ratio of SUC-DTX to HSA was increased to 100 to provide suitable amount of drug to react with positively charged NH$_2$ (97 sites, including lysine, arginine, and histidine residues in HSA) to increase drug substitution. Also, the amount of organic solvents used in preparation was reduced to < 10% of aqueous phase to save HSA from coagulation, and pH of conjugation medium was increased to 8.

The results showed that the total drug substitution based on bound and unbound DTX was about 1.3% which is the same as previous work, but 71% of DTX was attached to HSA which is 20% more than previous report (4).

Several methods were used to attach FA to HSA, but the most suitable method was reported. In one method, FA was activated by EDC and NHS in PBS 0.1 M, pH = 9, and HSA was added to solution of activated FA, but after adding SUC-DTX to attach DTX to HSA-FA conjugate, FA was detached. Thus, FA was activated in minimum amount of organic solvent and reacted by HSA solution in PBS. This conjugate can tolerate DTX attachment reaction. Depending
on the amount of FA which reacted by HSA, FA/HSA ratios were 30–40 (for 0.3 mmol FA) and 4–6 (for 18 μmol FA).

To achieve targeting properties and accessibility of FA, we need to attach more than 1 molecule FA to HSA. Based on previous reports 4–6 mole, FA was attached to each mole of HSA (21).

To synthesize DTX-HSA-FA conjugate, HSA-FA conjugate containing 4–6 mole FA per each mole of HSA was synthesized and reacted with activated SUC-DTX. The total drug content of DTX-HSA-FA conjugate based on bound and unbound DTX determined using HPLC method which was about 1.2%. In addition to DTX-HSA conjugate, 71% of drug was attached and FA/HSA ratio in final product was 4–6.

The best method which was successful for HSA-FA conjugate preparation was used for biotin attachment. Activated biotin was added to HSA solution (in PBS 0.1 M, pH = 9.1). Because of low solubility of biotin, the amount of organic solvent was increased to 34% of aqueous phase. The amount of biotin was determined by HABA/Avidin reagent kit, and results showed that 4–8 mole of biotin was attached to each mole of HSA.

For synthesis of DTX-HSA-biotin conjugate, SUC-DTX was activated by EDC and NHS and reacted with HSA-biotin conjugate containing 4–8 mole biotin per each mole of HSA. The total drug loading of DTX-HSA-biotin conjugate was as same as DTX-HSA-FA conjugate and biotin/HSA ratio in final product was 4–8.

**Characterization of nanoparticles**

Nanoparticle preparation method was developed to reduce multiple steps of reaction and fabrication methods which may affect physicochemical stability of HSA. Using current method by separate preparation of protein conjugate and target moiety attachment in different reaction provide a chance to prepare HSA-FA and DTX-HSA in maximum ratio compared to previous reported method and to mix them in suitable ratio to achieve proper amounts of DTX and FA in nanoparticles. Thus, effect of reaction variables on final product was reduced to minimum, and the method will more reliable for scale-up and industrial production. Achieved nanoparticles were in the size of 185 nm, PDI of 0.254, zeta potential of −21.7 mv (Figure 3A). These nanoparticles consist of 1.3% DTX and 4–6 mole FA per each mole of HSA. In addition, negative surface charge of these nanoparticles plays a major role in their stability in the medium. The more repulsion represents the more stable nanoparticle system. Negative charge on the NPs surface is due to carboxyl and folate groups.

DTX-HSA+HSA-biotin nanoparticles were in the size of 183 nm, PDI 0.149, Zeta potential −19.8 mv (Figure 3B). These nanoparticles consist of 1.3% DTX and 2–4 mole biotin per each mole of HSA.

In these drug delivery systems, drug was attached only by covalent bond to the human serum albumin and there is no physically loading. The use of targeting moieties can facilitate uptake of drug delivery system by cancerous cells and reduce the toxic effect of drug on normal cells. This approach actually is combination and complementary method which inspired from two commercialized approaches. The first one is Abraxane® which is a commercialize paclitaxel albumin bond nanoparticles that paclitaxel physically loaded in albumin nanoparticle (without covalent bonds), and the second one is folate-targeted medication such as Vintafolide® (folate-targeted vincristine commercialized by Endocyte and Merck Co, West Lafayette, IN, USA), which is a promising method for cancer treatment as well.

**Cell viability studies**

Three cancer cell lines were used to determine cytotoxicity of targeted and non-targeted nanoparticles and conjugates.

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**Table: Nanoparticle Characterization**

<table>
<thead>
<tr>
<th></th>
<th>Diam. (nm)</th>
<th>% Intensity</th>
<th>Width (nm)</th>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Peak 1</td>
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<td>94.4</td>
<td>119</td>
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<tr>
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<td>5.6</td>
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<td>Intercept</td>
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<td>B</td>
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<td></td>
<td></td>
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<tr>
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<td>100.0</td>
<td>85.8</td>
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<tr>
<td>Peak 2</td>
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</tr>
<tr>
<td>Intercept</td>
<td>0.501</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 3:** Z-average particle size and size distribution by intensity of (A) DTX-HSA+HSA-FA nanoparticles and (B) DTX-HSA+HSA-biotin nanoparticles. The graph shows partially monodispersed nanoparticles.
including MDA-MB-231 (human breast carcinoma cells, FR+), A549, (human lung adenocarcinoma, FR−), and 4T1 (murine breast carcinoma cells) which the latter overexpresses biotin receptors (+++) and folate receptors (+) (1). MDA-MB-231 (as FR+) and A549 (as FR−) cell lines were used to evaluate cytotoxic effects of DTX-HSA+HSA-FA nanoparticles and DTX-HSA-FA conjugate. Similar concentrations of free DTX (as positive control), DTX-HSA nanoparticles or DTX-HSA conjugate, respectively, were added to cell culture medium. Medium was used as negative control. Because of cytotoxic effects of DMSO as the solvent of DTX, stock solution of DTX was diluted 200 times by cell culture medium before adding to the cells to keep the amount of DMSO below 0.5%. Same concentration of DMSO was used as control to evaluate its cytotoxic effects as well.

As shown in Figure 4A, at 80 nM concentration, DTX-HSA+HSA-FA nanoparticles have around 20% less cell viability compared with DTX-HSA nanoparticles and around 30% less cell viability compared with free DTX. This results were subjected to statistical analysis by one-way ANOVA test and Tukey HSD as post hoc analysis (SPSS Statistic software, version 21; IBM Corporation, New York, USA), and the results have shown significant difference with p value < 0.5. Thus, it may be concluded that DTX-HSA+HSA-FA nanoparticles could increase cytotoxicity compared with free DTX and DTX-HSA nanoparticles in MDA-MB-231 (as FR+) due to targeting properties acquired by FA moieties. These results are more pronounce at higher concentrations (e.g. 160 nm) which can be correlated to more FA molecules on the surface of nanoparticles. 0.5% concentration of DMSO was used as control. Data showed that DMSO killed 15% of cells in MDA-MB-231 cell line. Thus, it could be concluded that 15% of cytotoxicity of free DTX is related to DMSO (it should mentioned DMSO only used for dissolving of DTX. Other conjugates and nanoparticles are soluble in water and DMSO did not used to dissolve them). At 80 nm concentration, DTX-HSA+HSA-FA nanoparticles have around 20% less cell viability compared with DTX-HSA nanoparticles and around 30% less cell viability compared with free DTX on MDA-MB-231 cell line. If 15% of cytotoxicity was related to DMSO, it could be concluded that cell viability of DTX-HSA+HSA-FA nanoparticles have around 45% less than free DTX. In the other word, cytotoxicity of DTX-HSA+HSA-FA nanoparticles is 45% more than free DTX which is statistically significant.

Cytotoxicity of DTX-HSA+HSA-FA nanoparticles also was evaluated on A549 (FR−) cells to confirm the effect of FA on increased cytotoxicity. The results (Figure 4B) shown that there is no significance difference between cell viability of DTX-HSA+HSA-FA nanoparticles and DTX-HSA nanoparticles and free DTX by p value < 0.5.

DTX-HSA-FA conjugate showed similar results (Figure 5A). DTX-HSA-FA conjugate had < 12% cell viability compared with DTX-HSA conjugate and < 26% compared with free DTX at 80 nm. This difference was significant with p value < 0.5. Thus, it may be concluded that DTX-HSA-FA conjugate could increase cytotoxicity compared to free DTX and DTX-HSA conjugate in MDA-MB-231 as well at higher concentrations.

Cytotoxic effects of DTX-HSA-FA conjugate had no significant difference with DTX-HSA conjugate and free DTX (p value < 0.5) in A549 (Figure 5B).

DTX-HSA+HSA-biotin nanoparticles and DTX-HSA-biotin conjugate were also treated on 4T1 cells. The results (Figures 4C and 5C) showed that cytotoxicity of DTX-HSA+HSA-biotin nanoparticles and DTX-HSA-biotin conjugate was significantly higher than free DTX and DTX-HSA nanoparticles or DTX-HSA conjugate, respectively (p value < 0.5).

In vivo antitumor efficacy and survival study

Murine breast cancer xenograft model was developed to evaluate the antitumor effect of the DTX-HSA-FA and DTX-HSA-biotin conjugates in vivo. The survival time and
tumor growth of the mice in each group were monitored. The survival rate of the 4T1-bearing mice has shown in Figure 6. The mice in control group were died with 25% dying within 15 days and 100% dying within 21 days. For the mice injected with Taxotere/C226 10 mg/kg, 50% of the mice died within 15 days. While for the mice administered with DTX-HSA-FA at 5 mg/kg of DTX equivalent conjugates, none of mice died before 15 days and for DTX-HSA-biotin conjugates at 5 mg/kg of DTX equivalent conjugates, only 17% of the mice died during 15 days of treatment. The median survival time was 19, 16.5 and 23 and 32.5 days for control, Taxotere®, DTX-HSA-FA conjugate, and DTX-HSA-biotin conjugate, respectively.

In the control group, only tumor was induced and PBS was injected. In free DTX group, after tumor induction, Taxotere® was administered via tail vein with dose of 10 mg/kg [it is less than maximum tolerable dose which was reported 40 mg/kg in healthy BALB/c mice (59)]. Mice in all groups were anesthetized by ether steam before injection.

All of conjugates were dispersed by suitable amount in sterile water for injection and centrifuged 10 min in 10,000 g to separate large particles. The supernatant is evaluated for DTX content and dosed based on DTX equivalent. The supernatant was sterile-filtered and injected via tail vein during 1 min.

In control group, tumors grew up well while signs of tumor necrosis seen in the middle of tumor after 3 weeks (Figure 7A).

Administration of free DTX by dose of 10 mg/kg only reduces the rate of tumor growth, so that the tumor size reached to $1834 \pm 100 \text{ mm}^3$ at 3th week which has significant difference with control group ($p < 0.05$) (Figure 7B). The results showed that Taxotere reduced the speed of tumor growth. It means that Taxotere has inhibition effect on tumor compared to control group, but it could not reduce the tumor size.

Administration of all of conjugates at the dose of 10 mg/kg (based on DTX content) leading to death of mice in < 4 days which may related to several reasons including rate of injection and intolerability to medicine. Thus, the administered dose was reduced to 5 mg/kg.

All conjugates were administered with dose of 5 mg/kg (base on DTX content). Despite of lower doses administration in both DTX-HSA-FA and DTX-HSA-biotin groups, after administration of first dose, the average tumor size was reduced from $110.6 \pm 12 \text{ mm}^3$ to $31.2 \pm 37 \text{ mm}^3$ in DTX-HSA-FA group and from $108.6 \pm 8 \text{ mm}^3$ to $55.8 \pm 36 \text{ mm}^3$ in DTX-HSA-biotin group. Tumors were totally cured and disappeared in both groups after second dose (Figure 7A,B).

Based on above-mentioned results, it might be concluded that DTX-HSA-FA and DTX-HSA-biotin conjugates as targeted drug delivery systems, with smaller amounts of the medicine could produce better therapeutic effects com-
pared to free drug. It might because of DTX-HSA-FA and DTX-HSA-biotin conjugates could reach tumor site by EPR effect and maintain the effective therapeutic concentration of DTX for a long period of time by sustained cleavage of the conjugates, also using these targeting moieties could cause to better uptake of conjugates and delivered more amounts of DTX to the cancer cells.

**Conclusion**

In this study, HSA was employed as a carrier to prepare a macromolecular drug conjugate. DTX was attached to HSA by a succinate linker which can break in acidic environment of tumor interstitium fluid by a sustained manner (4,22,60). FA and biotin are small nutrition molecules which cancer cells uptake them actively, thus attachment of these molecules on the surface of conjugates can facilitate cell uptakes of drug. FA has specific receptors on the surface of cancer cell which is different from normal cells, thus folate attachment can increase site specificity of conjugate. Folate-targeted medications were commercialized including four distinct FA-drug conjugates, EC145 Vintafolide, EC1456 (folate–tubulysin), EC0652 (for prostate cancer), EC20 Etarfolatide (for molecular imaging) entered to clinical trials for the treatment of cancer by Endocyte Company (West Lafayette, IA, USA).

Two methods were used in preparation of folate or biotin-targeted conjugates. In one method, DTX-HSA, HSA-FA, or HSA-biotin were prepared separately and combined together by an emulsification/solvent evaporation method which is inspired from nab (nanoparticle albumin bound) technology. Nab technology was developed by Abraxis Bioscience and commercialized for production of Abraxane® (Paclitaxel albumin-bound nanoparticles for injectable suspension) which got FDA approval for treatment of advanced breast cancer, non-small cell lung cancer, and pancreatic cancer. By preparing conjugates separately, HSA affected less by harsh condition of reaction and variation in the yield of reaction had lower effect on final product. We can make DTX-HSA and HSA-FA or HSA-biotin separately and mix them by proper ratio based on DTX and targeting moiety content to achieve uniform product based on DTX and targeting moiety content in industrial scales. Although these nanoparticles had good cytotoxicity in cellular studies, there is a doubt that these particles may dissociate in body. The other way is simultaneous attachment of DTX and FA or biotin covalent bonds to HSA. In this way, HSA tolerated more stresses and control

![Figure 7:](image)

**Figure 7:** (A) Comparison of antitumor efficacy between control, free DTX, DTX-HSA-FA, and DTX-HSA-biotin groups in BALB/c mice-bearing breast cancer, (B) percentage of growth rate in BALB/c mice-bearing 4T1 tumor xenograft.
of repeatability is more difficult in industry, but the results showed that the antitumor effect of these conjugates was much greater than the free drug and survival time in longer as well. Thus, they might be promising drug delivery systems for cancer treatment which may develop their method of manufacturing for large-scale production. The introduced drug delivery systems have 2 mechanism of targeting. First, the nanoparticles or conjugates were accumulated in cancerous tissue using by EPR effect and the concentration of them increase over there. Then, accumulated targeted drug delivery systems in interstitium mostly uptake by cancerous cells because of the presence of targeting moieties such as folic acid or biotin. Thus, the first goal which achieved by these drug delivery systems is change the distribution of drug. Because the drug is attached to carrier by covalent bonds; thus, it released less in blood circulation (pH = 7.4) and after permeation to cancerous tissue, the drug will release with sustained manner. In these drug delivery systems, drug was attached only by covalent bond to the human serum albumin and there is no physically loading. The use of targeting moieties can facilitate uptake of drug delivery system by cancerous cells and reduce the toxic effect of drug on normal cells. This approach actually is combination and complementary method which inspired from two commercialized approaches. The first one is Abraxane® which is a commercialize paclitaxel albumin bond nanoparticles that paclitaxel physically loaded in albumin nanoparticle (without covalent bonds), and the second one is Folate-targeted medication such as Vintafolide® (folate-targeted vincristine commercialized by Endocyte and Merck & Co), which is a promising method for cancer treatment as well.

Acknowledgments

This article is part of a PhD thesis has been performed in Nanotechnology Research Centre, Faculty of Pharmacy, TUMS. Financial support for this work was provided by a grant from Research Council of Tehran University of Medical Sciences. The authors gratefully acknowledge Hakim pharmaceutical Company for its support to do this project. In addition technical assistance of Faezeh Khosravi, Ms. Salamian, Ms. Mirzaie, Nastaran Hosseinifar, and Mr. Gholami is appreciated.

Declaration of Interest

The authors report no declarations of interest.

References


