Tumor stromal disrupting agent enhances the anticancer efficacy of docetaxel loaded PEGylated liposomes in lung cancer

Aim: Therapeutic efficacy of anticancer nanomedicine is compromised by tumor stromal barriers. The present study deals with the development of docetaxel loaded PEGylated liposomes (DTXPL) and to investigate the effect of tumor stroma disrupting agent, telmisartan, on anticancer efficacy of DTXPL. **Methods:** DTXPL was prepared using proprietary modified hydration method. Effect of oral telmisartan treatment on tumor uptake of coumarin-6 liposomes and anticancer efficacy of DTXPL was evaluated in orthotopic xenograft lung tumor bearing mice. **Results:** DTXPL (105.7 ± 3.8 nm) showed very high physical stability, negligible hemolysis, 428% enhancement in bioavailability with significantly higher intratumoral uptake. Marked reduction in collagen-I, MMP2/9 and lung tumor weight were observed in DTXPL+telmisartan group. **Conclusion:** Combination of DTXPL with telmisartan could significantly enhance clinical outcome in lung cancer.

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Lung cancer is by far the most common cause of cancer related mortality in both men and women. Non-small-cell lung cancer (NSCLC) accounts for 87% of total cases of lung cancer with overall 5-year survival rate less than 18.2% [1,2]. Depending on the stage of lung cancer and condition of patients, the chemotherapeutic regimen for lung cancer includes following drugs: Cisplatin/carboplatin or paclitaxel/docetaxel, etoposide, vinorelbine, erlotinib or other tyrosine kinase inhibitors, gemcitabine and pemetrexed. Despite recent advances in diagnosis and treatment, the clinical outcome amongst NSCLC patients is still not impressive. Docetaxel (DTX) is one the most widely used for drug for the treatment of various types of solid tumor including ovarian, breast, lung and head/neck cancers. Docetaxel has extremely poor water solubility and rapid precipitation tendency, which necessitates very high

concentration of ethanol and surfactant for its intravenous injection. Tween 80 in Taxotere® formulation is responsible for severe life threatening hypersensitivity and peripheral neuropathy [3,4]. A safer formulation to enhance patient compliance and therapeutic efficacy of DTX is always in demand. As a result, current research is mainly focused on developing nanomedicinal formulation such as liposomes, polymeric micelles, albumin nanoparticles, polymer drug conjugates, etc. Liposomes are the most versatile nanocarrier with superior biocompatibility and large scale manufacturing feasibility compared to other types of nanocarrier. Abraxane®, Doxil®, Depocyt®, Myocet®, DaunoXome®, Marqibo®, etc. are commercially available anticancer nanoformulations for parenteral administration. It is noteworthy that all of them are liposomal formulations except Abraxane® [5].

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In spite of enormous preclinical research on development of nanocarriers for taxanes and liposomal formulations for variety of drugs, there is no liposomal formulation of docetaxel available in the market. There are few research papers describing the parenteral delivery of DTX using PEGylated nanocarriers (PLGA, caprolactone or gold nanoparticle) and even fewer reports on docetaxel loaded PEGylated liposomes [6–9]. Surprisingly, none of the reports have demonstrated the physicochemical stability of DTX nanocarriers. Poor physical stability of DTX encapsulated liposomes is the major limiting factor for its clinical development. Usually, formulations developed for preclinical research purposes have stability sufficient enough to carry out animal studies but for clinical application, long term stability is indispensable. Therefore, an innovative liposomal preparation method is required to formulate DTX liposomes with an extended stability. PEGylated liposomes of DTX could potentially serve these purposes by improving the solubility of DTX without using ethanol or surfactant, enhancing the pharmacokinetic of DTX due to prolong plasma circulation and enhancing the anticancer efficacy because of enhanced permeation and retention (EPR) mediated higher intratumoral uptake.

Poor diffusion and penetration of drug into solid tumors owing to high interstitial pressure or tumor stromal barriers, is a serious issue limiting the efficacy of existing chemotherapeutic agents [10]. Leaky neovasculature, abnormal lymphatic system and perivascular fibrosis are associated with high interstitial fluid pressure. Matrix rigidity and fibroblast contractility in solid tumor result in increased fiber tension which leads to decreased convection and transcapillary transport of anticancer drugs to deep tumor regions. Therapeutic outcome of cancer chemotherapy has been significantly improved by use of nanocarriers due to their ability to delivery of anticancer drugs selectively to tumor tissues with limited accumulation in healthy tissues [5,10,11]. However, mere development of nanocarriers of chemotherapeutic drugs and reliance on EPR effect for its intratumoral uptake is not adequate to achieve desired anticancer effect. EPR mediated passive targeting and tumor surface antigen directed active targeting lead to higher accumulation of drug into tumor tissues [12]. However, irrespective of the surface characteristics and targeting nature of the nanocarriers, their distribution within solid tumor matrix is severely hindered by dense collagen network and very high interstitial tumor pressure due to highly fibrous interstitium in solid tumors. Collagen rich network embedded in hyaluronan of tumor stroma significantly impede the penetration of drug and nanoparticles into tumors. The extracellular matrix (ECM)

creates both a physical and hydrodynamic barrier in the form of high intratumoral pressure which prevents most therapeutics and nanoparticles from reaching the tumor foci [11,13,14]. Thus, disruption of tumor ECM is very important for enhanced delivery of drug and nanoparticles in solid tumors. Recently, there is a huge interest in drug or enzyme reducing the collagen density in tumor, which has the collateral effect of decreasing tumor interstitial fluid pressure (TIFP) thereby facilitating higher drug/nanocarrier penetration. Few researchers have showed enhanced uptake of antibody, virus or nanocarriers in tumor tissues by disrupting stromal barrier using bacterial collagenase, relaxin or TGF- β 1 inhibitors [15–17]. In our previous report, we have demonstrated that inhalable telmisartan reduced collagen level in lung tumors and enhanced uptake of fluorescent polystyrene nanoparticles. Telmisartan is an angiotensin receptor blocker used for the treatment of hypertension. In our earlier published work, we have studied that TGF- β 1 inhibition was the primary mechanism for antifibrotic activity of inhalable telmisartan in lung tumors [13].

In this paper, a modified hydration method was proposed to produce a stable DTX loaded PEGylated liposomes (DTXPLs) and compared with conventional liposomes. Thereafter, the effect of oral telmisartan treatment on tumor stromal disruption, uptake of liposomes in tumors and anticancer efficacy of DTXPLs in orthotopic lung tumor bearing athymic nude mice was evaluated.

Experimental

Chemicals & drugs

Docetaxel was purchased from AK scientific (CA, USA). Phospholipids were purchased from Lipoid (Germany). All other chemicals and HPLC solvents were purchased from Sigma-Aldrich (MO, USA). DMEM-F12K medium, fetal bovine serum and other cell culture materials were purchased from Lonza (Basel, Switzerland). Primary antibodies were purchased from cell signaling technology (USA).

HPLC analysis

Reverse phase HPLC-UV method was used to analyze DTX for stability, release and pharmacokinetic studies. Chromatographic separation was achieved on Waters 717 instrument equipped with waters symmetry (250 mm/4.6 mm/5 μ m) column using ACN:Phosphate buffer pH 3 (55:45) as mobile phase with flow rate of 0.55 ml/min, detected at 227 nm. Plasma DTX analysis was achieved at same chromatographic conditions. Retention time of DTX was 13.9 ± 0.15 min. Salting-out Liquid-Liquid Extraction (SALLE) method was used to extract DTX from plasma. For DTX extrac-

tion, 250 µl Acetonitrile was added to 150 µl plasma samples followed addition of 100 mg NaCl to separate acetonitrile and plasma layer. Samples were vigorously stirred and centrifuged at 5000 rpm for 10 min at 10°C. Supernatant was mixed with 50 µl of Milli-Q water. Samples were directly loaded to HPLC auto-sampler for analysis.

Animals

Sprague–Dawley rats were purchased from Harlan Inc. (Indianapolis, IN). The rats were housed and maintained in specific pathogen-free conditions in a facility approved by the American Association for Accreditation of Laboratory Animal Care (AALAC). Food and water were provided ad libitum to the animals in standard cages. Animals were maintained at standard conditions of 37°C and 60% humidity. All experiments were done in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Florida A&M University.

Female, 6-week old, athymic Nu/nu mice were purchased from Harlan Inc. (IN, USA). The mice were housed and maintained in specific pathogen-free conditions in a facility approved by the American Association for Accreditation of Laboratory Animal Care. Food and water were provided ad libitum to the animals in standard cages. Animals were maintained at standard conditions of 37°C and 60% humidity. All experiments were done in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Florida A&M University. Animals were acclimatized for 1 week prior to the tumor studies.

Preparation of liposomes

Liposomes were prepared using modified hydration method. Briefly, phospholipids, cholesterol, PEGylated phospholipid and DTX were dissolved in chloroform. The solution was drop wise added to mannitol at 45°C with constant stirring and left overnight for evaporation of chloroform. Resultant powder was dispersed into water and sonicated for 4 min using probe sonicator (Branson Probe Sonicator, USA). Molar ratio of DTX to phospholipids and processing parameters were optimized on the basis of physical stability. Taxotere was prepared using same formula as marketed docetaxel formulation – Taxotere® (Sanofi-Aventis, USA). For ethanol injection method, phospholipids and DTX were dissolved in absolute ethanol and injected into water at 55°C [18]. Batches of thin film hydration method were prepared by vacuum evaporating chloroform solution containing phospholipids and DTX in round bottom flask followed by hydration with water at 55°C.

Particle size, zeta potential & entrapment efficiency

Hydrodynamic diameter of formulation was analyzed by dynamic light scattering (DLS) using Nicomp PSS particle size analyzer (PSS Systems, USA). Formulation was diluted into HPLC grade water to achieve concentration of 0.1 mg/ml of DTX. Entrapment efficiency was analyzed using gel permeation chromatography using Sephadex G50 column (Sigma Aldrich, USA).

Scanning electron microscopy (SEM) analysis

The SEM pictures of liposomes were taken on a FEI Nova NanoSEM 400 machine fitted with a tungsten (W) filament. The air dried liposomes samples were mounted on a metal stub using double sided carbon tape followed by coating with 8 nm thick layer of iridium (Ir) by sputter coating for 30 min. Finally the pictures were taken at an accelerating voltage of 5 kV and observed using 3 µm scale bars.

In vitro release

DTX release from taxotere and liposomes was evaluated by a dialysis bag method using phosphate buffer pH 7.4 with 0.2% w/v tween 80 as release medium. The dialysis bag (MWCO 50 kD, Spectrum Lab, USA) filled with 1 ml of DTX formulation was tied to the paddle of USP dissolution apparatus I (50 rpm, 37°C). Samples were withdrawn at 0.5, 1, 2, 4, 8, 12 and 24 h and DTX content was analyzed by HPLC.

In vitro hemolysis study

In vitro hemolysis study was carried out using human red blood cells (RBCs) as per the procedure reported earlier [19]. Briefly, blood cells were separated from plasma by centrifugation at 5000 rpm for 10 min followed by discarding the supernatant and resuspending cell pellet into equivalent ml of HBSS to achieve the same hematocrit. Taxotere and DTXPL were dispersed in HBSS to prepare 0.5 mg/ml solution of DTX. Appropriate quantity of this stock solution was added to 1 ml of RBC dispersion to achieve 50, 25 and 10 µg/ml DTX concentrations. The samples were incubated for 30 min at 37°C and centrifuged at 5000 rpm for 10 min and the supernatants were subjected to analysis. Hemoglobin release was determined by UV spectrophotometer at 550 nm. HBSS was used as the negative control and sodium dodecyl sulphate solution was used as positive control (100% hemoglobin release). % hemolysis was calculated by following formula:

$$\% \text{ hemolysis} = \frac{(\text{absorbance of test sample} - \text{absorbance of negative control})}{(\text{absorbance of positive control} - \text{absorbance of negative control})} \times 100$$

Plasma-to-blood ratio

Plasma-to-blood ratio (PBR) of taxotere and DTXPL were analyzed in human blood [19]. Taxotere and DTXPL were diluted with phosphate buffer to prepare DTX concentrations of 250 µg/ml each. 20 µl of this solution was added to 980 µl of human blood to prepare final concentration of 5 µg/ml of DTX in blood (n = 6). After a 30 min incubation period at 37°C, three samples from each set were centrifuged (5000 rpm, 10 min) while other three samples were frozen at -80°C for complete hemolysis. Plasma was separated from centrifuged samples. DTX concentration in plasma and hemolysed blood was calculated by HPLC method.

In vivo pharmacokinetic in rats

In vivo pharmacokinetic study of taxotere and DTXPL was performed in Sprague–Dawley rats. After 1 week of acclimatization, animals were divided into two groups – six animals in each group. Taxotere and DTXPL were diluted with saline and 5 mg/kg DTX dose was administered via tail vein. Blood samples were collected via tail vein at 0.066, 0.5, 1, 2, 4 and 8 h post dosing. Blood samples were centrifuged at 10,000 rpm at 4°C for 10 min to separate plasma. DTX plasma concentration data after intravenous administration was analyzed by Kinetica 5® (ThermoFisher, USA).

Inoculation of A549 in mice

A549 cells were grown in DMEM-F12K Media containing 10% FBS and standard antibiotic mix at 5 % CO₂ and 37°C. For orthotopic lung tumor model, mice were anesthetized with isoflurane and a 5 mm skin incision was made to the left chest, 5 mm below the scapula. Hamilton syringe with 28-gauge hypodermic needles was used to inject A549 cells through the sixth intercostal space into the left lung. The needle was quickly advanced to a depth of 3 mm and removed after the injection of the cells (2 million per mouse) suspended in 100 µL PBS (pH 7.4) into the lung parenchyma. Wounds from the incisions were closed with surgical skin clips. Animals were observed for 45 to 60 min until fully recovered. The animals develop lung tumor within 14 days after the cell inoculation [2,20,21].

Antitumor study

After 14 days, animals were randomly divided into four groups (Saline solution, Taxotere, DTXPL and DTXPL + telmisartan) of 12 animals each. Telmisartan treatment was started on 15th day of cell inoculation while DTX treatment was started from 17th day. Dose of DTX (intravenous) and telmisartan (oral) was 5 mg/kg body weight. Six doses of DTX formulations were given biweekly for 3 weeks. Two doses of telmis-

artan were given at 24 h and 48 h before each DTX dose. Six animals from each group were sacrificed (carbon dioxide inhalation), 3 days after the last dose of DTX formulation. Lungs were collected, weighted and observed for lung tumor growth using a stereomicroscope (Olympus, USA). The same tumors were used for immunohistochemistry (IHC) and western blot analysis. Study was continued till 45 days with remaining animals to observe the survival rates over the period of time.

Coumarin-6 liposome uptake in lung tumor

After 21 days of A549 inoculation, animals were divided into two groups: control and telmisartan. Control group received saline solution and telmisartan group received two doses of telmisartan (5 mg/kg, once a day) by oral route. On 24th day, both the group received coumarin-6 liposomes (1 mg/kg) via tail vein injection. Animals were sacrificed (carbon dioxide inhalation) after 1 h and lungs were collected. Cryosection (40 µm thickness) of part of lung bearing tumor nodules was carried out and immediately observed under fluorescence microscope. Intensity of green color as a measurement of coumarin-6 uptake in normal lung and tumor tissue was carried out using ImageJ software. The tumor sections were also stained with picosirius red (Abcam, USA) to evaluate the collagen density.

Western blot analysis

Protein samples for western blot analysis were extracted from lung tumor tissues using RIPA lysis buffer containing protease inhibitors and 500 mM phenyl methyl sulfonyl fluoride (PMSF). Protein concentration was estimated using BCA protein assay reagent kit. Equal amounts (50 µg) of protein from different groups were denatured by heating for 5 min in SDS sample buffer and separated using 10% SDS-PAGE. The protein samples separated on SDS-PAGE were transferred to nitrocellulose membranes for immunoblotting. After blocking, the membranes with skim milk (5% skim milk in 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.5% Tween 20) probed with primary antibodies. The primary antibodies such as survivin, MMP-2, MMP-9 and β-actin were used in 1:1000 dilutions. HRP-conjugated secondary antibodies were used. The detail procedure for western blotting was followed according to our published methods.

Immunohistochemistry of CD31 expression

Paraffin-embedded tumor tissues were deparaffinized and blocked for peroxidase activity for CD31 expression. The specimens were washed with PBS twice and then incubated in normal goat serum according to the

manufacturer's instructions (Santa Cruz Biotechnology, CA, USA). Subsequently, the sections were incubated with CD31 antibody, at 4°C overnight, followed by a 30 min treatment with HRP secondary antibody (Santa Cruz Biotechnology). After three washes with PBS, the sections were developed with diaminobenzidine-hydrogen peroxidase substrate and images were taken microscopically. The number of CD31 positive endothelial cells and/or the number of blood vessels were counted in four different high power fields per section (400×). The average number of microvessels per high power field was examined as described earlier [22].

Statistics

All data are presented as the mean $\pm$ standard deviation (SD). The significance of difference in treatment groups was determined using Student's *t*-test or one-way ANOVA using GraphPad prism version 5.0 (CA, USA), where value of *p* < 0.05 between the groups was considered as statistically significant difference between these groups.

Results

Formulation development & characterization

Physical instability within short period of time and poor drug loading are the two major issues in developing formulation of DTX. Therefore, our focus was to develop a novel formulation with high DTX loading and long term physical stability. Results of liposomal DTX batches prepared using various methods are given in [Table 1](#). Initially, methods of liposome preparation were compared and optimized on the basis of physical stability. Ethanol injection and thin film hydration are most widely used and industry accepted methods of manufacturing liposomes for various preclinical and clinical studies. As shown in [Table 1](#), DTX-loaded liposomes prepared using ethanol injection showed very rapid precipitation of DTX while thin film hydration showed marginally better stability depending on type of phospholipids used.

Unsaturated phospholipid (DOPC) showed fivefold higher stability compared with the liposomes prepared using saturated lipid (DPPC). However, particle size of liposomes prepared using both the lipids were nearly similar. Liposomes prepared using DOPC gave particle size of 132.6 ± 12.7 nm while DPPC gave slightly lower size of 114.2 ± 8.5 nm. DTXPL prepared using modified hydration method showed significantly higher stability compared with conventional methods like ethanol injection and thin film hydration. Initially, on dispersion into water, 20–30 μ m sized multilamellar liposomes were formed. Ultrasonication has significantly reduced the liposome size in the range of 100

nm. Effect of cholesterol, mannitol, and type of phospholipids, hydration temperature, DTX loading and DTX concentration on physical stability of DTXPL was analyzed to optimize the formulation.

Amount of cholesterol has critical role in DTX precipitation. Batch prepared using DOPC and 3 mole percent DTX with 15 mole percent of cholesterol showed maximum stability for 3–4 days as shown in [Table 1](#). Batches with same composition prepared using higher or 0% cholesterol content showed lesser stability. Cholesterol content above 40 mole percent of the batch showed rapid precipitation within 2–3 h. Thus, further batches were prepared using 15 mole percent of cholesterol, in other words, DTX:cholesterol weight ratio around 1:3 was optimized. We have observed that approximately 100 nm sized DTX liposomes formed after sonication step were stable for longer period of time when passed through sephadex G50 column. Extraliposomal mannitol was adversely affecting the physical stability of liposomes. Mannitol free batches showed stability for 3–4 days while unprocessed batches were found to be stable for 2 days only. Physical stability data mentioned in [Table 1](#) represent the results of various batches after mannitol separation. Batches prepared with saturated phospholipids like DSPC and DPPC become unstable within 2–4 h, while batches prepared with DOPC (unsaturated phospholipid) were much more stable. Hydration temperature of proliposomal powder composed of phospholipid, DTX, cholesterol and mannitol was playing crucial role. Batches hydrated with water at 10 and 25°C showed nearly similar stability for up to 2–3 days while the batches hydrated at 45°C showed higher physical stability for 4 days.

DTX loading was found to be the most critical parameter affecting the stability. Drastic reduction in stability was observed with small increment in DTX loading from 3 to 5 mole percent as shown in [Table 1](#). All the batches described in [Table 1](#) have final DTX concentration of 1 mg/ml. Batches with higher DTX concentration 1.5 and 2 mg/ml with same DTX mole percent loading showed inferior stability compared with 1 mg/ml batches. The effect of final DTX concentration on physical stability was less significant compared with DTX loading. Various excipients – solubilizer, surfactant, salt and albumin were added in DTXPL to investigate the effect of those excipients on stability. List of excipients and concentration are described in [Table 1](#). Surprisingly, all the excipients were found to be adversely affecting the stability, in other words, facilitating the DTX precipitation rate instead of slowing it down. Therefore, an alternative strategy was explored to enhance the stability of parent DTXPL. Blank liposomes (BL) of DOPC and DSPC were prepared using ethanol injection method. Appro-

Table 1. Composition and results of DTXPL batches prepared using different methods.

Method	DTX loading (mole percent)	Phospholipid	Excipient added after DTXPL preparation	Physical stability at room temperature
Ethanol injection	3	DOPC	–	Immediate precipitation
Ethanol injection	3	DPPC	–	Immediate precipitation
Thin film hydration	3	DOPC	–	Stable for 10–12 h
Thin film hydration	3	DPPC	–	Stable for 2–3 h
Modified hydration	3	DPPC	–	Immediate precipitation
Modified hydration	3	POPC	–	Stable for 12 h
Modified hydration	3	DOPC	–	Stable for 4 days
Modified hydration	4	DOPC	–	Precipitated within 24 h
Modified hydration	5	DOPC	–	Precipitated within 4 h
Modified hydration	3	DOPC	TPGS (0.5% w/v) Sodium Citrate (0.5% w/v) Hydroxy propyl β cyclodextrin (0.2% w/v) PEG 400 (1% w/v) Tween 80 (0.5% w/v) Albumin (1% w/v)	Reduced the stability of parent liposomes. Formed large agglomerates of DTX
Modified hydration	3	DOPC	Blank liposomes of DOPC	Stable for 2 weeks 7–8-fold enhancement in stability
Modified hydration	3	DOPC	Blank liposomes of DSPC	Stable for 6–7 days twofold enhancement in stability

appropriate amount of BL was added to DTXPL containing 3.5 mole percent DTX at 1.2 mg/ml DTX concentration to achieve final loading of 3 mole percent DTX at 1 mg/ml DTX concentration. Addition of DSPC-BL led to twofold enhancement in stability while addition of DOPC-BL resulted in 7–8-fold higher stability at room temperature. Particle size and entrapment efficiency was not significantly affected by drug loading and drug concentration. We observed that physical stability was roughly 8- to 10-times higher at 2–8°C than room temperature for all the batches. Finally, optimized formulation was composed of mixture of DTXPL+DOPC-BL with DTX loading of 3 and 1 mg/ml DTX concentration. It was found to be physically and chemically stable for 2 months at 2–8°C. The average particle size and zeta potential of finally optimized batch was 105.7 ± 3.8 nm and -12.4 mV, respectively. Entrapment efficiency was found to be 97.4%, which confirmed that DTX was confined to liposomal bilayers.

The SEM analysis of the liposomes revealed almost spherical morphology and the structures observed were in agreement with SEM images of liposomal formulation reported earlier [23]. Interestingly, SEM image of the liposomes prepared with thin film hydration

methods showed large crystals (Figure 1B) of DTX while no such crystals were observed in SEM images of liposomes prepared using modified hydration method (Figure 1A).

In vitro release

Sustained release of DTX was one of the desired parameters. As shown in Figure 2, taxotere showed complete release of DTX within first 2 h whereas DTXPL showed sustained release of DTX over the period of 48 h. Release of DTX from taxotere was following first order release kinetic while DTXPL exhibited 0 order release profile. DTXPL showed mere 40% DTX release in 48 h. However, complete release is expected *in vivo* due to degradation of liposomes in extracellular and intracellular milieu *in vivo*.

In vitro hemolysis study and PBR

Effect of DTXPL formulation on Human RBC was evaluated by incubating various concentration of formulation with RBC suspension. Both hemolysis and redispersion of RBC were evaluated and compared with taxotere. As shown in Figure 3A, negligible hemolysis was observed even at highest concentration of DTX. Further, very quick and complete redispersion of

RBC suggested that DTXPL did not alter the surface characteristics of RBC (Figure 3B & D). Contrary, taxotere formulation showed 22.7% hemolysis at 25 $\mu\text{g}/\text{ml}$ DTX concentration and more importantly redispersion of RBC was very poor on inversion (Figure 3C). Taxotere showed PBR of 1.09 ± 0.08 while DTXPL showed higher PBR of 1.42 ± 0.12 . It has indicated that liposomes were intact and DTX was preferentially confined to liposomal bilayer instead of partitioning into RBC.

Pharmacokinetic study

The plasma concentration profiles and pharmacokinetic parameters of DTX after intravenous injection of taxotere and DTXPL are depicted in Figure 4 and Table 2, respectively. As expected, there was a significant difference in pharmacokinetic profile of DTX. Noncompartment, one compartment and two compartment analysis of pharmacokinetic profile was carried out to understand the distribution and elimination pattern of liposomal DTX versus taxotere. Noncompartment analysis showed that the MRT of taxotere (1.99 h) was much low when compared with the DTXPL (8.08 h). DTXPL showed very low clearances rate of $0.35 \text{ (mg)/}(\mu\text{g/ml})/\text{h}$ when compared with taxotere, $1.63 \text{ (mg)/}(\mu\text{g/ml})/\text{h}$. Liposomal formulation showed higher C_{max} and AUC compared with taxotere which is a micellar formulation of DTX. Importantly, DTXPL showed 428% enhancement in bioavailability

of DTX compared with taxotere. Long circulation and extended release of DTX from liposomes have collectively contributed to higher AUC. In one compartment analysis of the plasma concentration vs time data, the drug loaded liposome cleared faster from plasma than the taxotere, which suggested that the liposomes are getting distributed toward the more perfused organs. MRT of DTX was lower for DTXPL (0.35 h) compared with taxotere (0.53 h). The two compartment analysis demonstrated that the DTXPL were quickly removed from the circulating system ($\text{Cl} = 0.33 \text{ (mg)/}(\mu\text{g/ml})/\text{h}$) with rapid distribution ($t_{1/2 \text{ alpha}} = 0.044 \text{ h}$) and slow terminal elimination phase ($t_{1/2 \text{ beta}} = 7.83 \text{ h}$) compared with taxotere ($t_{1/2 \text{ alpha}} = 0.35 \text{ h}$ and $t_{1/2 \text{ beta}} = 3.27 \text{ h}$).

Uptake of coumarin-6 PEGylated liposomes in lung tumor

Coumarin-6 PEGylated liposomes were injected to observe their distribution in normal lung vs tumor tissue. Cryosection of lungs of control and telmisartan treated animals are shown in Figure 5A. Intensity of green fluorescence in normal lung tissue is nearly equal in both the groups but it was significantly higher in tumor in telmisartan treated group. Further, green fluorescence intensity of normal lung part and tumor part calculated using ImageJ software has clearly suggested the enhanced uptake of coumarin-6 in telmisartan treated group (Figure 5B).

Figure 1. Scanning electron microscopy analysis of DTX-loaded liposomes prepared using modified hydration method (A) and thin film hydration method (B).

Figure 2. *In vitro* release of DTX from taxotere and DTXPL. Release study was carried out in USP apparatus II using dialysis bag (MWCO 50 kD) in phosphate buffer pH 7.4 with 0.2% w/v tween 80 at 37°C.

Anticancer study in orthotopic lung tumor bearing athymic nude mice

Picrosirius red stained of lung sections of control and telmisartan treated animals are shown in Figure 6A. Control showed bright red collagen fibers while reduced amount of red fibers were observed in telmisartan treated group. As shown in Figure 6B, lungs were completely covered with tumor growth in control group while partially covered in taxotere and DTXPL treated group. Interestingly, significant inhibition of tumor growth was observed in DTXPL + telmisartan group. The same results were reflected in lung tumor weight analysis (Figure 6C). There was no statistically significant difference observed in the tumor growth and lung tumor weight between taxotere and DTXPL treated animals. Results of taxotere and DTXPL were very surprising because both the group showed marginal improvement and nearly

similar anticancer efficacy despite of significantly different in their pharmacokinetic profile. Oral telmisartan did not show any inhibition of lung tumor growth (data not shown). Interestingly, lung tumor weight of DTXPL in oral telmisartan pretreated group was 14 and 10.87-fold lower than control and only DTXPL treated group, respectively. Moreover, lung integrity was mostly intact and regular in DTXPL+telmisartan treated animals. Results of survival study were in complete agreement with tumor inhibition data (Figure 6D). DTXPL+Telmisartan group showed 80% survival at the end of 45 days, while other groups showed less than 50% survival at the same time. All the animals in control group showed complete mortality within 40 days.

CD31 immunostaining confirmed the significantly lower microvessel density in combination group compared with DTXPL alone group (Figure 7A). Western blot analysis further confirmed the superior anticancer and antifibrotic effects of DTX+Telmisartan liposome compared with DTX alone. Immuno blot analysis for survivin expression suggested that the antiapoptotic marker survivin was decreased in DTXPL+telmisartan group (Figure 7B). Quantitative analysis of western blot images suggested that in DTXPL and DTXPL+telmisartan groups showed 0.91- and 2.10-fold reduction of survivin expression compared with untreated control tumors. Significant ($p < 0.01$) down regulation of MMP9 and MMP2 expression was observed in DTXPL+telmisartan treated tumors (Figure 7C & D).

Discussion

This is the first report demonstrating the improved delivery of DTX liposomes to lung tumors by using oral telmisartan as an antifibrotic agent. Development of long circulating DTX liposomes with extended stability profile is very challenging task. DTXPLs were

Figure 3. *In vitro* hemolysis study of DTXPL at various DTX concentrations was carried out on human RBC. DTXPL showed no hemolysis (A) and no aggregation on redispersion (B) even at very highest concentration of DTX. Taxotere showed hemolysis (C) and aggregation of RBC (D) at 25 µg/ml DTX concentration.

successfully prepared using an innovative modified hydration method developed in our laboratory. The physical stability of DTXPLs was successively increased by exploring various strategies with simultaneously understanding their role in modulating the stability of liposomes. There is no previous report investigating the pharmacodynamics profile of PEGylated liposomal DTX against orthotopic lung tumor bearing athymic nude mice. Second, collagen rich tumor stroma poses a significant hurdle in efficient delivery of nanocarrier to the majority of the tumors. Only long plasma circulation is not enough to deliver the drug to deeper layers of lung tumor. Therefore, in present study, telmisartan was used as an oral antifibrotic agent to disrupt the tumor stroma to enhance the permeation of DTXPLs.

Ethanol injection and thin film hydration are widely adopted methods reported in literature. However, DTX liposomal batches prepared by both the methods showed very poor physical stability. Both the drugs of taxane class, paclitaxel and docetaxel are very difficult to solubilize and stabilize in aqueous system [3,24]. DTX is quite a bulky molecule compared with the head-chain structure of phospholipids. The insertion of DTX into the bilayer would take more space and push the phospholipid molecule to be closer to each other. However, intermolecular force between lipids would try to resist the insertion of DTX. This intermolecular force rises with more DTX incorporated into the lipid bilayer. Feng *et al.* have performed studies on interaction of paclitaxel (DTX is a semisynthetic derivative of paclitaxel) with phospholipids and reported that paclitaxel is miscible with the lipid but there is a repulsive interaction between them. Our results with DTXPL are in agreement with their hypothesis [25].

Noteworthy, novel modified hydration method developed in our lab is very suitable to entrap such molecules with very poor dynamic and kinetic solubility, for example, paclitaxel, docetaxel. In modified hydration method, the lipid film is prepared over mannitol and the duration of hydration step is much shorter (1 min) compared with thin film hydration method (45 min). However, separation of soluble carrier, mannitol, is an additional step required in modified hydration method. We can hypothesize that distribution of DTX in inner

Figure 4. Pharmacokinetic profile of taxotere and DTXPL in SD rats (n = 6). DTXPL showed higher plasma concentration of DTX at all the time points.

leaflet of liposome bilayer could be higher when prepared using modified hydration method compared with thin film hydration method. Bilayer packing and membrane curvature of small unilamellar vesicles (SUVs) result in a large area and low density of the outer layer while the inner layer has increased packing density [26]. Inner layer is less hydrated/polar compared with outer layer and therefore drug entrapped within outer layer is transferred to aqueous layer much faster than drug entrapped within the inner layer [26,27]. High amount of mannitol or resultant osmotic pressure might be responsible for inner layer favored distribution of DTX during hydration step. However, presence of high amount of mannitol in external phase led to enhance precipitation rate and therefore mannitol was immediately removed after sonication step. Liposomes prepared using DOPC showed better stability compared with DPPC. DOPC forms less ordered bilayer with high cross sectional area due to presence of double bond which might help in encapsulating DTX for longer period of time. Also, possibility of interdigitation-mediated drug precipitation is negligible with DOPC compared with DPPC [28].

Though, modified hydration method produced DTX liposome with improved stability compared with conventional methods, the stability was not sufficient enough for clinical purpose. The main reason behind

Table 2. Pharmacokinetic parameters.

	Taxotere	Liposomes
C _{max} (µg/ml)	6.1 ± 1.7	10.68 ± 2.9
AUC (µg.h/ml)	5.29 ± 1.1	22.65 ± 4.3
T _{1/2} (h)	6.67 ± 1.6	7.39 ± 1.8
MRT	1.99 ± 0.36	8.08 ± 2.5
Relative BA	–	428%

Figure 5. Distribution of coumarin-6 liposomes in tumor bearing lung. (A) Fluorescence images of lung sections of control and telmisartan treated animals. Red arrow indicates the tumor region. (B) Green fluorescence intensity in normal and cancerous lung was calculated and expressed as mean $\pm$ SD. p-values were calculated using *t*-test and $p < 0.05$ considered as significant. Normal lung tissue showed similar fluorescence but telmisartan treated group showed higher fluorescence in tumor region as shown in graph.

destabilization is the transfer of DTX from liposomal bilayer to aqueous phase, where it leads to aggregation and precipitation of DTX at very rapid rate. We have hypothesized that addition of various solubilizer, surfactant or albumin may reduce the amount of free DTX in aqueous phase and attenuate the precipitation process but results were completely opposite to our hypothesis. Presence of these excipients in the external phase of DTXPL, resulted in more hydrophilic and hydroxyl group rich environment. Thus, additions of hydrophilic excipients were found to adversely affect the stability by promoting the transfer in aqueous phase followed by rapid precipitation. DTX can be transferred to other liposomes by collision between

the liposomes or by passive diffusion through aqueous phase [29,30]. Based on this, we hypothesized that addition of blank liposomes might be helpful in inhibiting DTX precipitation. There was a remarkable enhancement in stability of DTXPL was observed after addition of blank liposomes of DOPC or DSPC. Blank DOPC liposomes showed even better stability profile compared with DSPC blank liposomes. Difference in membrane fluidity and permeability due to difference in phase transition temperature (DOPC: -17°C ; DSPC: 55°C) of both the lipids could be the main reason for this behavior [26]. Blank liposomes proved to be the most appropriate reservoir system to maintain sink conditions, irrespective of DTX transfer mecha-

Figure 6. *In vivo* studies in A549 orthotopic lung tumor bearing nude mice. **(A)** Picrosirius red staining of lung tumor of control and telmisartan treated animals. Telmisartan treated animals showed significant reduction in collagen I fibers. **(B)** Orthotopic growth of untreated and treated xenografts. Lungs were collected 3 days after the last dose of DTX formulation. Lung integrity was severely compromised in untreated group. **(C)** Lung tumor weight (mg $\pm$ SD) of control and various treatment groups. **(D)** Kaplan–Meier survival plot.

nism. At both the storage conditions, in other words, room temperature and 2–8°C, DSPC liposomes are in gel phase indicating condensed and structurally well organized-somewhat rigid arrangement of hydrocarbon chains. However, DOPC liposomes are in liquid crystalline phase at both the storage conditions. We can assume that reversible transfer of DTX between DTXPL and DOPC blank liposomes could be easier than DSPC blank liposomes.

The negative surface charge of DTXPL could be contributed by negatively charged phosphate group of PEG-DSPE which is in accordance with the previous report [31]. Sustained release of DTX from stable and long circulating liposomes is extremely important to achieve EPR dependent passive targeting of tumor [32–34].

The pharmacokinetic profile of DTX liposomes was very much in correlation with the data of *in vitro* release profile and blood to plasma DTX ratio. The longer $t_{1/2}$ in the PEGylated liposomes is related to the reduced uptake of liposomal drug by the elements of the reticuloendothelial system (RES) [12]. Prolong

circulation enhance the chance of extravasation in leaky tumor vasculature and also provide long term exposure of drug to cancer cells. Taxotere maintained the therapeutic concentration of DTX only for short duration while DTXPL released drug in a controlled manner which maintained the DTX concentration within therapeutic window for prolonged period. The lower V_{ss} of DTXPL suggested that the liposomes controlled the distribution of DTX to unwanted tissues and resulted in long circulation in blood stream.

Another important aspect of this work was to investigate the effect of tumor stroma disruption (reduction in tumors fibrosis) on permeation of PEGylated liposomes and efficacy of DTXPL. Therapeutic benefit of EPR effect mediated tumor uptake of nanocarriers is limited by poor penetration to deeper regions in tumor. Of course, EPR helps in preferential passive uptake but further access into tumor tissue is minimized by tumor stroma. It was reported that stromal depletion results in improved tumor perfusion and a lower TIFP [35]. We have already demonstrated that telmisartan admin-

Figure 7. Immunohistochemistry (IHC) and western blot analysis of lung tumor. (A) IHC of lung tumor section for microvessel density using CD31 staining. CD31 stained tumor section of DTXPL+telmisartan treated animal showed marked reduction in microvessels. **(B)** Western Blot analysis of orthotopic lung tumor lysates. Western blot images of different protein expressions in tumor lysates. Quantitative analysis of expressions of **(C)** MMP9 and **(D)** MMP2. * $p < 0.05$; ** $p < 0.01$.

istered by inhalation route showed higher accumulation of fluorescent polystyrene nanoparticle in tumor region [12]. Since, oral route has very high patient compliance over aerosol based inhalation route, in the pres-

ent study telmisartan was administered by oral route. Further, use of fluorescent PEGylated liposome (Coumarin-6 as fluorescent probe) could be a superior choice over fluorescent polystyrene nanoparticles. Therefore,

use of coumarin-6 loaded PEGylated liposomes to investigate tumor uptake is more relevant than using fluorescent polystyrene nanoparticles. Higher uptake of coumarin-6 can be correlated with the disruption of collagen network and tumor stroma by telmisartan. In this report for the first time we are showing simultaneous distribution of PEGylated coumarin-6 liposomes between healthy and cancerous tissue. Though there are several reports describing the use of coumarin-6 liposomes to investigate the cellular uptake but these studies have been limited to *in vitro* imaging only [36–38].

The restrictive tumor microenvironment can be ‘normalized’ by pretreatment of agents that interfere with vascular growth factors (e.g., TGF- β , VEGF, etc.) or agents promoting the degradation of ECM components [13]. In our earlier report, we have shown that telmisartan reduced the expression of Collagen-1, TGF- β 1, cleaved caspase-3, Vimentin and E-Cadherin expression in lung tumor [13]. In this paper, we have investigated the effect of telmisartan treatment on level of MMPs. In solid tumors, MMPs expression is regulated by TGF- β , while MMPs are produced either by cancer or stromal cells, activate latent TGF- β in the ECM, together facilitating the enhancement of tumor progression. MMPs are mostly involved in the recycling of ECM as well as in releasing cytokines and growth factors from ECM. Tumor stromal cells express high levels of MMP2 at the advancing tumor front and are responsible for invasion and metastases. Although, there are homologies between functional domains among the MMPs, only MMP2 and MMP9, contain collagen binding domains (CBDs) formed by three-tandem fibronectin type II-like modules. MMP2 is a key component of cancer cell migration across collagen by a mechanism involving CBD-mediated MMP2 interactions with collagen. As shown in results, DTX+telmisartan combination significantly inhibited the MMP2 and MMP9 expression. As mentioned earlier, tumor extracellular matrix (ECM) is mainly composed of a dense collagen network embedded in the gel of glycosaminoglycans (GAGs), primarily hyaluronan. A number of studies demonstrating the use of collagenase, hyaluronidase and lysyl oxidase to degrade tumor ECM, as a means to improve the delivery of various therapeutics have been published in last decade [39,40]. Collagenase labeled gold nanoparticle exhibited 35% higher tumor accumulation compared with unlabeled gold nanoparticles [17]. Also, it has been shown that liposomes injected 1 h after the collagenase injection, led to approximately 1.5-fold increase in tumor uptake. Further, pretreatment with collagenase and hyaluronidase were found to enhance the transfection efficiency of plasmid DNA in subcutaneous tumor [39].

However, orally bioavailable small molecular inhibitors should be preferred over intravenous injection of

proteolytic enzymes for disrupting the tumor stroma. Recently, losartan has been reported to reduce stromal collagen and hyaluronan production, associated with decreased expression of profibrotic signals including TGF- β 1 [41]. There are few small and large molecular (lerdelimumab and metelimumab) TGF- β inhibitors currently under preclinical or clinical trial showing antifibrotic activity in cancer, glaucoma and other fibrosis related disorders [42]. Liu *et al.* reported that TGF- β inhibition improves the distribution and efficacy of therapeutics in breast carcinoma by normalizing the tumor stroma [15]. In a pancreatic tumor model, a small molecule TGF- β receptor I inhibitor (LY364947) showed enhanced uptake of the nanoliposomes–Doxil® and polymeric micelles (30 and 70 nm), but did not affect distribution of small molecular weight chemotherapeutic agent [43]. Further, TGF- β type I receptor inhibition resulted in decreased pericyte coverage of the endothelial cells in tumor neovasculature and promoted higher accumulation of nanomedicines [43]. Most of these studies reported the use of novel TGF- β inhibitor while we have used a low dose of a well-established drug to minimize the risk of possible side effects and toxicity. Simultaneous delivery of PEGylated formulation with antifibrotic therapy was further reflected in orthotopic xenograft tumor bearing nude mice model. Significantly higher tumor survival, intact lung structure and lung tumor weight similar to normal mice at the end of animal study clearly indicated the promising potential of DTX+telmisartan treatment. Superior pharmacodynamics activity was contributed by oral telmisartan mediated enhanced uptake of DTXPL in tumor region over healthy lung tissue. The strategy of using tumor stromal disrupting agents prior to the treatment with anticancer drug loaded nanocarrier has a promising translational potential. Therapeutic efficacy of various types of nanomedicine (liposomes, polymer-drug conjugates, polymeric micelles) can be significantly enhanced by disrupting tumor stroma using a small molecule antifibrotic agent. Since there are many nanomedicinal products for the delivery of drugs, DNA, siRNA, diagnostic agents or imaging agents will probably be commercialized in the near future; this approach has potential clinical applications. Moreover, DTXPL has potential for clinical application due to its facile and scale up feasible technology, long term stability compared with liposomal formulations and enhanced efficacy of docetaxel.

Future perspective

Pretreatment with antifibrotic agent prior to the treatment of anticancer nanomedicine will be integral part of cancer chemotherapy in future. Nanomedicine is future of cancer treatment and most of the nanoformu-

lations under preclinical or clinical trial are designed for taxane (paclitaxel or docetaxel) delivery. In such scenario, there is a need to explore means to enhance the delivery of nanoparticle to deeper layer of tumors. Therapeutic efficacy of various types of nanomedicine (liposomes, polymer–drug conjugates, polymeric micelles) can be significantly enhanced by disrupting tumor stroma using a small molecular antifibrotic agent. Further, our novel modified hydration method has huge scope of commercialization for clinical use due to its several advantages over conventional method.

Conclusion

Modified hydration is a superior method to prepare stable liposomes of drugs with severe solubility and precipitation issues. Addition of small amount of blank liposomes leads to remarkable enhancement in physical stability of DTXPL. This method has a huge scope for commercialization due to its facile and scale up feasible manufacturing technology, long term stability and high drug loading capacity. PEGylation driven prolong circulation, improved pharmacokinetics, EPR facilitated passive accumulation and telmisartan mediated deeper penetration of DTXPL have collectively contributed to superior anticancer efficacy of DTX. Enhancement of intratumoral deposition of nanocarrier by oral telmisartan treatment could be a simple and extremely beneficial approach in improving the clinical outcome of existing and upcoming anticancer nanoformulations.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Preparation of docetaxel-loaded PEGylated liposomes using modified hydration method

- Docetaxel liposome prepared using modified hydration method (developed in our laboratory) showed very high stability compared with liposomes prepared using conventional (ethanol injection or thin film hydration) methods.
- Modified hydration technique was found to be the most suitable method to develop stable PEGylated liposomes of drug with severe solubility and precipitation problem, for example, paclitaxel or docetaxel.

Strategies to prevent docetaxel precipitation

- Blank liposomes composed of unsaturated phospholipids serve as a reservoir system to subside the docetaxel precipitation rate while hydrophilic solubilizers and surfactants enhanced the docetaxel precipitation.

Pharmacokinetic studies in rats

- The pharmacokinetic profile of DTX liposomes was very much in correlation with the data of *in vitro* release profile and blood to plasma DTX ratio. The longer $t_{1/2}$ in the PEGylated liposomes is related to the reduced uptake of liposomal drug by the elements of the reticuloendothelial system (RES).

Uptake of coumarin-6 PEGylated liposomes in lung tumor

- Significantly higher uptake of liposomes in tumoral vs healthy tissues was observed in telmisartan pretreated animals suggesting the compromised tumor stromal barrier in telmisartan treated animals.

Anticancer study in orthotopic lung tumor-bearing nude mice

- Picrosirius red staining showed a significantly lower collagen I in the tumor of telmisartan-treated animals.
- Significantly higher tumor survival, intact lung structure and lung tumor weight similar to normal mice at the end of animal study clearly indicated the promising potential of DTX+Telmisartan treatment.
- Superior pharmacodynamics activity is contributed by telmisartan mediated TGF- β and other cytokines modulatory effects in disrupting tumor ECM and enhancing DTXPL uptake in tumor region over healthy lung tissue.

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