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RESEARCH PAPER

Is an Alternative Drug Delivery System Needed for Docetaxel? The Role of Controlling Epimerization in Formulations and Beyond

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ABSTRACT

Purpose The presence of 7-epidocetaxel in docetaxel injection and *in vivo* epimerisation has been reported to be the cause for development of tumor resistance to chemotherapy including docetaxel by inducing tumor cell protein cytochrome P450 1B1. The objective of this study was to determine systemic toxicity of Taxotere® containing 10% 7-epidocetaxel and to develop PEGylated liposomal injection that could resist epimerization *in vivo*. Another need for PEGylated liposomal delivery of docetaxel is to avoid reported hypersensitivity reactions of marketed products like Taxotere® and Duopafei® containing high concentration of tween-80.

Methods The PEGylated liposomes loaded with docetaxel were prepared using thin film hydration method. The *in vivo* toxicity of Taxotere® containing 10% 7-epimer was studied in B16F10 experimental metastasis model.

Results B16F10 experimental metastasis model using C57BL/6 mice injected with Taxotere® containing 10% 7-epimer showed higher weight loss as compared to Taxotere® containing no epimer at single dose of 40 mg/kg indicating higher systemic toxicity. Incubation of PEGylated liposomes with phosphate buffer saline (pH 7.4) containing 0.1% w/v Tween-80 for 48 h showed better resistance to docetaxel degradation when compared with Taxotere® injection indicating better *in vivo* stability of liposomal docetaxel. In addition, PEGylated liposomes showed enhanced *in vitro* cytotoxicity, against A549 and B16F10 cells, than Taxotere®.

Conclusion We can therefore expect less *in vivo* conversion of liposomal loaded docetaxel into 7-epimer, more passive targeting to tumor tissues, decreased 7-epimer induced systemic toxicity and tumor resistance to chemotherapy compared to Taxotere®. Further *in vivo* studies are needed to ascertain these facts.

KEY WORDS 7-epidocetaxel · adenocarcinoma cell line · docetaxel · mouse melanoma cell line · PEGylated liposome · Taxotere®

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INTRODUCTION

Docetaxel (DTX) was approved by the FDA for the treatment of advanced ovarian cancer in April 1994 and later in December 1999 for the treatment of patients with locally advanced or metastatic non-small cell lung cancer (Taxotere®, Rhone-Poulenc Rorer, now Sanofi Aventis). Taxotere® (TXT) is approximately twice as potent as taxol in inhibiting cold and calcium-induced depolymerization of microtubules (1). It contains a taxane derivative in association with ethanol and polysorbate 80. This composition is proved to be fairly unstable and shows a significant degradation, expressed in the formation of 7-epidocetaxel (EDTX) when exposed it to heating (2). The EDTX, a well known degradation product of DTX (3), was first quantified in the blood and urine of patients under chemotherapy with TXT (4). The mechanism of DTX degradation was reported to be pH dependent. In the acidic media or in the presence of electrophilic agents, D ring as well as B ring opening and/or rearrangement will occur with DTX (Fig. 1) whereas, in the basic media, the cleavage of ester groups at positions 2, 4, and/or 13 will occur. One of the principal paths of degradation is the epimerization of hydroxyl group at position 7 which results in the formation of EDTX by way of retro aldol reaction. The degradation of DTX can result in products which have reduced activity or are completely inactive. Further, these products demonstrate pharmacological and toxicological profiles completely different from DTX (5).

Bornique and Lemarie investigated the interaction of DTX and its epimer, EDTX with recombinant human cytochrome P450 1B1 (hCYP1B1) which is present in various human tumors and is postulated to be responsible for the development of resistance by tumor cells towards chemotherapeutic agents, including DTX (6). They studied the effect of DTX on rhCYP1B1 mediated 7-ethoxyresorufin O-deethylase (EROD) activity. Naphthoflavone (1 μM), a CYP1B1 inhibitor, totally inhibited the EROD activity. The DTX at 3, 10, and 30 μM did not show major effects on EROD activity. However, at 100 μM, DTX increased EROD activity by 3.8 folds. Additionally, they have shown that EDTX, which is in equilibrium with DTX as a minor compound in solutions, was a potent activator of rhCYP1B1 with a >7-fold increase of EROD activity at 10 μM. These results confirm that EDTX is a potent inducer of this enzyme. Therefore, it is desirable to minimize or eliminate the presence of EDTX in the pharmaceutical formulations containing DTX and/or its trihydrate (7). In addition, the above reports instigate a pharmaceutical scientist, not only, look into control of the degradation of DTX into EDTX in pharmaceutical products during manufacturing and storage but also look into its *in vivo* stability after infusion.

It has been surprisingly found that, the addition of an organic and/or inorganic acid induces a significant increase in the stability of DTX in injection even after heating at higher temperature (2). The acids selected were citric acid, acetic acid, formic acid, ascorbic acid, aspartic acid, benzoic acid, hydrochloric acid, sulphuric acid, phosphoric acid, tartaric acid, glutamic acid, lactic acid, maleic acid or succinic acid. The most preferred acid is citric acid anhydrous as defined in the European Pharmacopoeia 2007. Therefore, the viable strategy is to develop long circulating DTX liposomes which protect the drug in their lamellae from degradation while in the blood circulation (by not releasing the drug during circulation) for long time. Also, they release the drug only at target site for effective chemotherapy with less chance of developing tumour resistance.

In addition, the limited clinical application of DTX was also attributed for its poor aqueous solubility (7 μg/mL) (8,9), low bioavailability and high toxicity. Presently used Taxotere® and Duopafei® contain high concentration of non-ionic surfactant tween-80 and the adverse reactions reported in patients (*e.g.*, hypersensitivity, fluid retention, neurotoxicity, musculoskeletal toxicity and neutropenia) are presumed to be due to either the drug itself or tween-80 (10). Thus, in order to eliminate tween-80 based vehicle the alternative dosage forms such as liposomes (11,12), cyclodextrin complex (13), polymeric nanoparticles (14), micelles (15), solid lipid nanoparticles (SLN) (16) and nanostructured lipid carriers (NLC) (17) have been developed. Among these dosage forms, the lipid-based nanocarriers (liposomes, NLC and

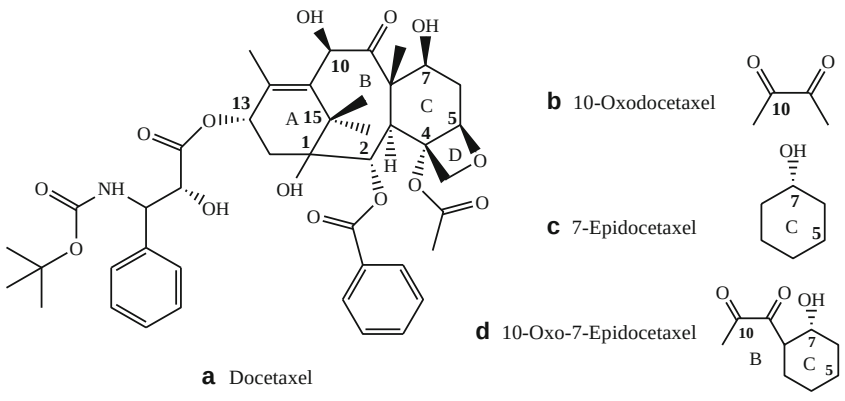

Fig. 1 Chemical structures of (**a**) Docetaxel and its degradation impurities (**b**) 10-oxodocetaxel, (**c**) EDTX and (**d**) 10-oxo-7-epidocetaxel.

SLN) showed potentially favourable characteristics such as improved drug dispersibility, enhanced drug solubilisation, enhanced drug transmembrane transport capability and increased therapeutic efficacy with reduced toxicity.

In the present study we have prepared DTX loaded PEGylated liposomes composed of mixture of phospholipids (HSPC, DPPC and DPPG) as combination of more than one lipid can increase the hydrophobic drug loading (18,19). DTX loaded liposomes were prepared using combination of saturated phospholipids, having high Tg, at high temperature of hydration and annealing in the presence of organic acid added as degradation inhibitor. The prepared PEGylated liposomes were tested for percentage drug loading and DTX degradation that occur during preparation. The DTX degradation during *in vitro* drug release study was undertaken in order to predict the *in vivo* degradation of DTX into EDTX. The prepared PEGylated liposomes were also tested for *in vitro* cytotoxicity and cell uptake character using human lung adenocarcinoma cell line (A549), to predict the performance of our prepared formulations in humans, and mouse melanoma cell line (B16F10), to predict the performance of our prepared formulations during preclinical study (mouse B16F10 lung melanoma model). The *in vitro* uptake of prepared liposomes against rat liver normal cell line (K9) was also studied to predict the *in vivo* liver and spleen uptake during preclinical study.

MATERIALS AND METHODS

Chemicals

Docetaxel (purity: 99.3%; total impurity:0.4%), 7-epidocetaxe and 10-oxo-7-epidocetaxel were kindly gifted by Fresenius Kabi Oncology Limited, GURGAON, India. Fully Hydrogenated Soya Phosphatidylcholine (HSPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DDPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylglecerol (DPPG) and DSPE-mPEG$_{2000}$ were kindly gifted by Lipoid GMBH (Ludwigshafen, Germany). Cholesterol Extra Pure, Citric acid, Tartaric acid, Maleic acid, Ascorbic acid and Vitamin E were purchased from Sigma Aldrich, Mumbai, India. Dialysis bag (12000 MWCO) was purchased from Himedia Lab, Mumbai, India. BCA kit was purchased from Bangalore Genei, Bangalore. All other chemicals used were of analytical reagent grade and were used without further purification.

Cell Culture

B16F10, mouse melanoma cell line and A549, human lung adenocarcinoma cell line were purchased from NCCS, Pune, India. The rat normal liver cell line, K9, was purchased from American type culture collection (ATCC), USA. The B16F10, A549 and K9 cells were maintained in Iscove's Modified Dulbecco's Media (IMDM), RPMI-1640 (GIBCO) and F12K (Sigma), respectively. The media was supplemented with 10% heat inactivated foetal bovine serum, FBS (GIBCO) and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin). Cultures were maintained at 37°C in 5% CO_2 humidified atmosphere.

Preparation of Conventional Liposomes

The negatively charged conventional liposomes (CLs) composed of lipid mix, HSPC/DPPC/DPPG/CHOL (0.08991: 0.08991:0.0116:0.0522 mM), DTX (0.0058 mM; 5 mg) and degradation inhibitor tartaric acid (400 μg) were prepared by Thin Film Hydration Technique as described by Bangham (20) (Fig. 2).

Formation of Thin Lipid Film

The lipid phase, DTX and tartaric acid were weighed accurately and dissolved in chloroform and methanol solvent mixture (2:1 ratio, v/v). The round bottom flask containing solution was then attached to rotary evaporator (BUCHI Rotavapor R-200, BUCHI India Private Ltd, Mumbai, India), evacuated and rotated at 120 rpm in a thermo stated water bath maintained at 45 ± 1°C. The process was allowed to continue until all solvent had evaporated and a dry thin lipid film had deposited on walls of the flask. The flask was rotated under vacuum for additional 30 min after the dry residue was first appeared. Subsequently, the flask was kept overnight under vacuum to remove residual solvents.

Hydration of Lipid Film

The dry lipid film was hydrated with double distilled water (5 mL) at above glass transition temperature (85 ± 1°C) for 30 min. Then, the liposomal dispersion was transferred to volumetric flask, diluted with double distilled water and annealed by keeping in a water bath maintained at 85 ± 1°C for about 1 h.

Production of Small Unilamellar Vesicles (SUVs)

To prepare SUVs, the obtained multilamellar vesicles were probe-sonicated (Sartorius, LABSONIC® M, New York, USA) for 1 min (6 cycles of 10 s each at 80% amplitude and 0.6 duty cycle). After each cycle the dispersion was kept at 85 ± 1°C for 2 min that helps to reform liposomes after probe sonication. The sonicated liposomal dispersion was centrifuged at 6000 rpm for 10 min to separate unloaded drug and the supernatant was analyzed for % DTX content, % impurities generated, average mean particle size and zeta potential (analysed using Malvern Zetasizer Nano, NanoZS, Malvern Instruments, UK).

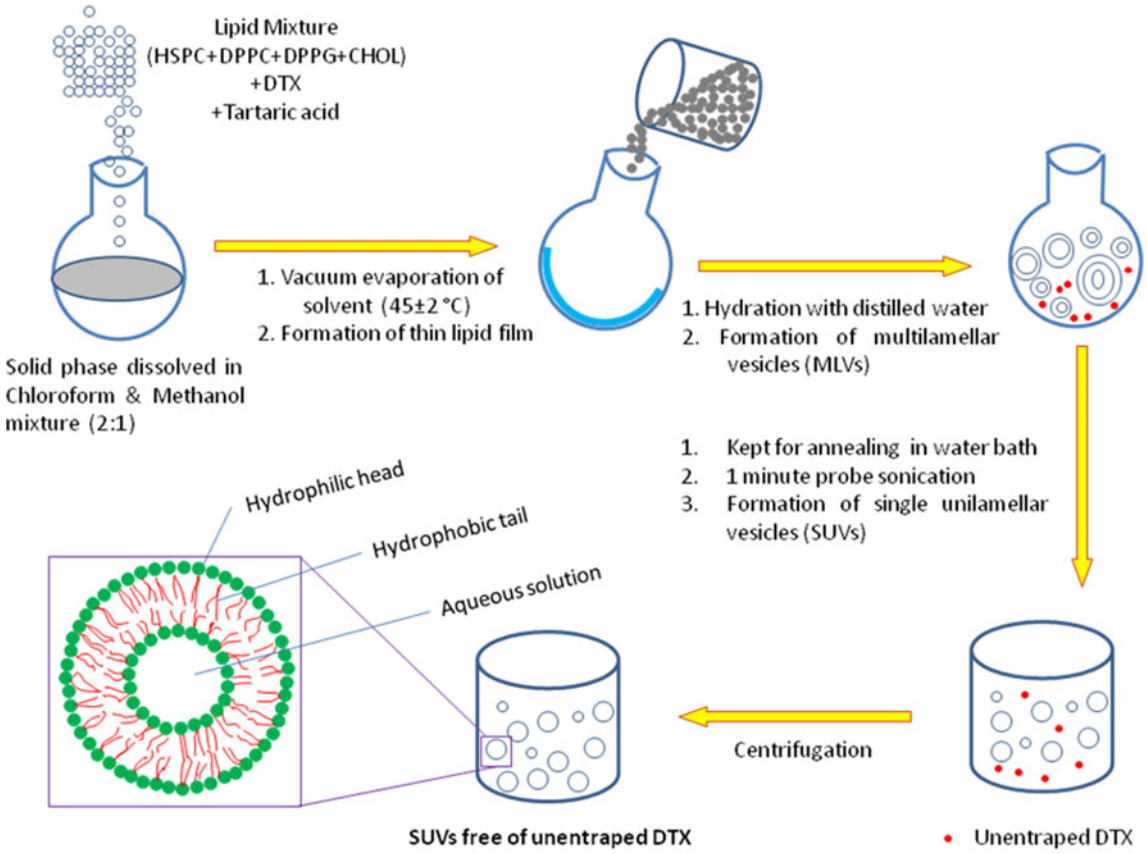

Fig. 2 The schematic view of the steps involved in liposome preparation.

Optimization of Impurity Free DTX-Loaded CLs

Preliminary experiments were carried out in the absence of organic acids (Data not shown) to optimize few formulation and process variables such as drug:lipid ratio, HSPC: DPPC ratio, hydration volume, cholesterol concentration, hydration temperature and annealing time. These variables were optimized based on particle size and DTX loading (analysed using UV-visible spectroscopy against blank containing the same concentration of lipids at 230 nm) of prepared CLs.

The optimized liposomal formulation prepared in the absence of organic acid showed significant amounts of DTX impurities (10-oxodocetaxel, EDTX and 10-oxo-7-epidocetaxel). In order to quantify the impurities formed at different temperature, liposomal formulations containing no organic acids were prepared by maintaining drug to lipid ratio of 1:30, cholesterol concentration of 0.0522 mM and conducting the hydration and annealing step at 3 different temperatures [60, 75 and 85°C for a total period of 1.5 h (includes hydration, annealing and during probe sonication)]. Prepared formulations were analysed for DTX content and its impurities generated using RP-HPLC (Shimadzu LC-20AT, Japan) and as per reported methods (21,22). The chromatographic condition includes mobile phase: acetonitrile:water (60:40, v/v); Kromacil C18 column (150×4.6 mm, 5 µm, Merck); flow rate: 1 mL/min; column temperature: 30°C; UV-visible detector; λ_{max}: 231 nm. The drug content and impurity content of the prepared liposomes were calculated using the below mentioned formulas.

$$\%\ \text{Drug content} = \frac{\text{Actual amount of drug entrapped in liposomes}}{\text{Actual amount of drug used for liposome preparation}} \times 100$$

$$\%\ \text{Impurity content} = \frac{\text{Actual amount of impurity entrapped in liposomes}}{\text{Actual amount of DTX used for liposome preparation}} \times 100$$

3^3 Factorial Design

Based on results obtained in preliminary experiments, cholesterol concentration (X1), hydration temperature (*X*2) and citric acid concentration (X3) were found to be the major variables affecting % drug loading and % impurity generation. Hence, 3^3 full factorial design was applied to find the optimized condition for higher drug content and minimum and/or absence of degradation impurities. Twenty-seven batches of different combinations were prepared by taking values of selective variables X1, *X*2 and X3 at different levels keeping other formulation and process variables invariant throughout the study. The prepared batches were evaluated for % drug content, % impurities generated and pH of the

Table I 3^3Full Factorial Design Consisting of Experiments for the Study of Three Experimental Factors in Coded and Actual Levels with Experimental Results

Formulation	Actual value variables			Response value		
	X1 (mM)	X2 (°C)	X3 (mg)	% Drug content	% EDTX formed	pH of formulation
CA-AL-1	0.0232	75	0.4	42.58 ± 3.76	–	5.4
CA-AL-2	0.0232	75	1	26.74 ± 1.93	–	4.65
CA-AL-3	0.0232	75	2	11.44 ± 1.46	–	4.01
CA-AL-4	0.0232	80	0.4	51.82 ± 2.81	1.676 ± 0.143	5.55
CA-AL-5	0.0232	80	1	26.1 ± 2.74	1.023 ± 0.124	4.52
CA-AL-6	0.0232	80	2	26.92 ± 1.0	–	3.93
CA-AL-7	0.0232	85	0.4	57.62 ± 1.92	1.786 ± 0.234	5.27
CA-AL-8	0.0232	85	1	49.36 ± 3.24	1.462 ± 0.098	4.45
CA-AL-9	0.0232	85	2	37.41 ± 2.67	–	3.76
CA-AL-10	0.0348	75	0.4	40.12 ± 1.11	–	5.55
CA-AL-11	0.0348	75	1	24.16 ± 2.29	–	4.41
CA-AL-12	0.0348	75	2	21.9 ± 2.22	–	3.82
CA-AL-13	0.0348	80	0.4	40.52 ± 1.87	1.243 ± 0.057	5.4
CA-AL-14	0.0348	80	1	28.8 ± 2.26	0.923 ± 0.034	4.56
CA-AL-15	0.0348	80	2	21.92 ± 2.98	–	3.88
CA-AL-16	0.0348	85	0.4	46.62 ± 3.78	1.388 ± 0.121	5.23
CA-AL-17	0.0348	85	1	38.44 ± 3.33	1.116 ± 0.099	4.42
CA-AL-18	0.0348	85	2	35.58 ± 1.67	–	3.75
CA-AL-19	0.0522	75	0.4	27.08 ± 1.74	–	5.01
CA-AL-20	0.0522	75	1	20.98 ± 2.44	–	4.35
CA-AL-21	0.0522	75	2	14.612 ± 2.78	–	3.71
CA-AL-22	0.0522	80	0.4	31.1 ± 0.98	0.434 ± 0.034	5.22
CA-AL-23	0.0522	80	1	21.22 ± 1.66	–	4.36
CA-AL-24	0.0522	80	2	13.12 ± 2.55	–	3.72
CA-AL-25	0.0522	85	0.4	49.64 ± 3.12	0.558 ± 0.079	5.12
CA-AL-26	0.0522	85	1	37.86 ± 2.45	–	4.37
CA-AL-27	0.0522	85	2	36.14 ± 3.87	–	3.91

Values are Mean ± SD, n = 3

CA-AL citric acid containing anionic liposomes

formulation as dependent variables. Table I summarizes the experimental runs and factor combinations employed along with translation of their coded levels into units. The results of dependent variables are recorded in Table I and that of reduced factorial design in Table II.

Comparison of Different Organic Acids as Degradation Inhibitors

The efficiency of other organic acids such as ascorbic acid, maleic acid and tartaric acid in controlling degradation impurities was also tested at molar equivalent quantity of citric

Table II 2*3 Factorial Design to Study the Influence of Three Experimental Factors (X_1, X_2 and X_3) in Coded Levels on % Drug Loaded (Y_2) in Liposomal Batches

Formulation code	X1	X2	X3	X1.X2	X1.X3	X2.X3	X1.X2.X3	Y2[a]
CA-AL-1	−1	−1	−1	+1	+1	+1	−1	42.6
CA-AL-19	+1	−1	−1	−1	−1	+1	+1	27.1
CA-AL-7	−1	+1	−1	−1	+1	−1	+1	57.6
CA-AL-25	+1	+1	−1	+1	−1	−1	−1	49.6
CA-AL-3	−1	−1	+1	+1	−1	−1	+1	11.4
CA-AL-21	+1	−1	+1	−1	+1	−1	−1	146
CA-AL-9	−1	+1	+1	−1	−1	+1	−1	37.4
CA-AL-27	+1	+1	+1	+1	+1	+1	+1	36.1
Calculated Effect[b] (%)	−3.67	+49.27	−40.73	+0.07	+4.73	+0.49	−1.04	

[a]Values rounded to one decimal point

[b]Calculated by the method of Yetes

acid concentration optimized. The efficiency of Vitamin-E (as antioxidant) in controlling impurity was also tested by replacing citric acid. The organic acid, which exhibited better controls over the impurity formation and improved the drug loading was identified and used in the final formulation.

Preparation of PEGylated Liposomes

PEGylated liposomes (PLs) were prepared by pre-insertion technique using thin film hydration method. The 1 mol%, 3 mol% and 5 mol% DSPE-mPEG$_{2000}$ were co-dissolved along with other phospholipids during film formation and the other steps followed are same as described above for CLs preparation. Prepared PLs were evaluated for pH, mean particle size, zeta potential, % DTX loading and % impurities loading. They were also tested for their steric stability using *in vitro* serum protein adsorption study.

Transmission Electron Microscope (TEM) Analysis

A drop of PEGylated (5 mol%) liposomal formulation was placed on a coated carbon grid and air dried. The samples were stained with 5 μL of 2.5% uranyl acetate for 30 s and air dried. The grid was then examined immediately under Philips Electron Microscope (Philips, Japan). The electron micrographs were obtained after magnifications. The physical characteristics of the particles observed by TEM were determined using selected area diffraction (SAD) technique. The measurement conditions were; $\lambda = 0.0251$ A^0 radiation generated at 200 kV as X-ray source with camera length of 100 cm.

Differential Scanning Calorimetry (DSC) Analysis

Thermal properties of plain DTX, physical mixture of DTX and other lipids (HSPC, DPPC, cholesterol and DSPE-mPEG2000, 1:1 w/w) and freeze dried DTX loaded PLs were investigated using Differential Scanning Calorimetry (METTLER, STARe SW 9.20, Mettler Toledo GmbH, Switzerland). Accurately weighed samples (7.2-11.5 mg) were placed in hermetically closed aluminum pans and empty aluminum pan was used as a reference. The samples were heated at a heating rate of 100°C per minute in the range of −50°C to 250°C under inert nitrogen atmosphere (23,24).

In Vitro Serum Protein Adsorption Study

To 300 μL of non-PEGylated and PEGylated liposomal suspensions in a 2 mL eppendorf tubes, 1000 μL of 100% foetal bovine serum was added and the liposome/serum mixture (76.9% final serum concentration) was incubated in a shaker incubator (Scigenic ORBITEK, Germany) for 1 h at 37°C. The liposomes were then separated from incubation mixture (1300 μL) through Sepharose CL-4B column (10 mL pipette loaded with Sepharose CL-4B gel) and eluted with double distilled water. The fractions with highest lipid content determined by Stewart method (25) (Supplementary Material), typically fractions 7, 8, 9 and 10 from each column, were pooled and analysed for mean particle size, zeta potential, total lipid content (Supplementary Material) and the amount of total serum proteins associated (26). Quantification of serum proteins in pooled liposomal fractions was performed using BCA Protein Assay Kit at the enhanced protocol (60°C for 30 min). The lipid in the sample did not interfere with protein assay under our experimental conditions. The protein binding index (P_B: grams of total protein/mol of total lipid) was calculated as per earlier report (27).

In Vitro Drug Release Study

Release of DTX from PLs was studied using the dialysis method at room temperature (28) and was compared with marketed TXT (Sanofi Aventis). The DTX release from PLs composed of HSPC alone and DPPC alone was also studied. The TXT and PLs equivalent to 1 mg of DTX were placed in dialysis tubes (MWCO 12000) and tightly sealed. Then, the tubes were immersed in 50 mL of release medium, *i.e.*, PBS (pH 7.4) containing 0.1% (w/v) tween-80 to maintain sink condition (28–30). While stirring the release medium using the magnetic stirrer at 150 rpm/min, the samples (1 mL) were withdrawn at predetermined time intervals (0.5, 1, 2, 4, 6, 10, 24 and 48 h) from the release medium and the same volume was replaced with the fresh medium. The 1 mL of sample was centrifuged at 5000 rpm for 5 min and the supernatant was bath sonicated for 5 min and analysed using RP-HPLC method as described before. The concentration of DTX released with respect to time was then calculated. The concentration of DTX remain unreleased from formulations was determined by dissolving dialysis bag contents in methanol after release study in order to check the mass balance. The stability of DTX in release medium was also studied by determining the amount of DTX impurities formed in the release medium at each time point. Also, we determined the amount of impurities remain unreleased from the formulations after release study.

In Vitro Cytotoxicity Assay

The cytotoxicity of TXT, DTX loaded CLs and PLs against A549 and B16F10 cell lines was determined using MTT dye reduction assay (31,32). Briefly, 4×10^3, 2×10^3 and 1.5×10^3 cells per well were seeded into 96-well plates for 24, 48 and 72 h study, respectively. The cells were incubated at 37°C in a 5% CO_2 incubator for 24 h, during which the cells were attached and resumed to grow. The 10% serum containing

media was removed from the wells and DTX formulations (in logarithmic dilution), serially diluted in media containing 10% serum, (0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM) were added to wells in quadruplicate (100 μL each). Control wells were treated with equivalent volumes of DTX-free complete media. After 24 h, 48 h and 72 h the supernatant was removed and each well was washed twice with 100 μL of PBS. The MTT solution (5 mg/mL solution in PBS pH 7.4) was diluted to 1 mg/mL in complete media and then 100 μL was added to each well and incubated overnight. The plates were then centrifuged for 20 min at 1500 rpm (Rota 4R-V/fm; Plasters Crafts Industries Ltd., Mumbai, India) and the supernatant was discarded. 100 μL of DMSO was then added to dissolve the MTT formazan crystals. Plates were shaken for 2 min and absorbance was read at dual wavelengths of 540/690 nm using the microplate reader (Molecular Devises, SPECTRUM AX190). The main reason for the dual wavelengths is that formazan crystals are being measured at 540 nm while the background absorbance (for the cells) is being measured at 690 nm. This background absorbance is then substracted from 540 nm. The IC_{50} values were then calculated graphically (logarithmic scale) by plotting % viability *versus* concentration. The % viability was calculated by considering the optical density of the control well as 100% viable (33).

In Vitro Cell Uptake Study Using Flow Cytometer

0.2×10^6 cells/well were seeded into 12 well plate and allowed to adhere and grow overnight. The cells were then treated with 1 mL of media containing 6-coumarin loaded CLs and PLs, equivalent to 25 μg and 50 μg of total lipid, for 30 and 60 min. Cells were washed thrice with PBS and harvested. The cells were then fixed using 1% PFA for 10 min and then washed twice with wash buffer (1X PBS containing 1% FBS and 0.01% sodium azide). Acquisition was done using flow cytometer (FACSCalibur™, BD Biosciences, USA). A minimum of 10,000 cells were acquired for each experimental set up and the results were analysed using BD CellQuest Pro software (v5.2.1).

Acute Toxicity Study

Animals

Female C57BL/6 mice (6–8 weeks old), ranging from 18 to 22 g were provided by Animal Care Facilities, ACTREC, Tata Memorial Centre, Navi Mumbai. All *in vivo* experiments were approved by the Institutional Animal Care and Use Committee. All care and handling of animals was performed with the approval of institutional review board of animal experiments.

Method

Acute toxicity study was conducted in B16F10 experimental metastasis model (34–36) to compare the acute toxicity of TXT injection alone and with 10% EDTX impurity after a single i.v. dose (37,38). The 6 to 8 week-old female C57BL/6 mice were injected with 0.1×10^6 cells/100 μL PBS intravenously. Mice were randomly assigned into 3 groups (6 mice/group): (A) Untreated control (PBS); (B) TXT treated; and (C) TXT with 10% EDTX treated. The mice were dosed at 40 mg/kg (approximately equivalent to human maximum tolerance dose) of TXT and TXT containing 10% EDTX on day-9 after B16F10 cell inoculation. The EDTX (formulated as like TXT injection) equivalent to 10% was mixed with TXT and injected into the mice *via* tail vein. The animals were weighed throughout the experiment and the dead ones were recorded. These data sets were used as indicators of systemic toxicity of TXT alone and with 10% EDTX. The mice were sacrificed on day-20 and the lungs were excised, photographed and fixed in 10% formaldehyde solution until use. The lung sections were fixed, stained with hematoxylin and eosin and observed under phase contrast microscope (Axio Imager. Z1 upright microscope, Germany).

RESULTS AND DISCUSSIONS

Optimization of Impurity Free DTXLoaded CLs

Liposomes were prepared using phospholipid mixture, HSPC/DPPC/DPPG/Chol, as the use of more than one phospholipid would increase the hydrophobic drug loading due to imperfect packing of lipid with varied dimensional characteristics (18,19). Also, the use of saturated phospholipids like HSPC and DPPC, having Tg value above 37°C, and cholesterol could show the enhanced *in vivo* stability of prepared liposomal injections than the liposomes composed of unsaturated phospholipids and no cholesterol (US patented Miradocetaxel injection) (39). The HSPC: DPPC ratio was optimized (1:1) for maximum drug content and minimum average particle size by preparing liposomes at different ratios of HSPC and DPPC keeping DPPG (0.011602 mM) and cholesterol (0.011602 mM) concentration as constant. The presence of negatively charged phospholipid (DPPG) decreased the liposomal size during probe sonication to nanometer range and also enhanced the suspension stability at 4°C. The drug to lipid ratio of 1:20, hydration temperature of 70–75°C, hydration volume of 3–5 mL, annealing time of 2–3 h and probe sonication time of 1 min were considered optimum for maximum DTX loading (93±3%) and minimum particle size (95±5 nm; PDI:0.249±0.036). The cholesterol concentration of 0.0348 mM was considered optimum for maximum drug loading (83±3%), minimum particle size (110±8 nm;

PDI: 0.275±0.024) and minimum drug leakage (10±2%) during 2 weeks of storage at 4°C. Our experimental results shows increased drug loading and enhanced *in vitro* suspension stability of liposomes composed of both HSPC and DPPC.

The HPLC analysis of liposomal suspension was carried out to resolve, identify and quantify the DTX impurities (10-oxodocetaxel, EDTX and 10-oxo-7-epidocetaxel). The retention time and relative retention time of standard DTX and its impurities are shown in Fig. 3. The DTX elute was first followed by 10-oxodocetaxel, EDTX and 10-oxo-7-epidocetaxel under the condition of analysis which indicated that the impurities are more lipophilic than the DTX. The order of lipophilicity of eluted constituents was 10-oxo-7-epidocetaxel > EDTX > 10-oxodocetaxel > docetaxel. These results are in accordance with the sequence of lipophilicity reported previously by Reddy *et al.* (40). Particularly, the impurity separated just after DTX and before EDTX has showed RT (5.51±0.073 min) and RRT (1.143) values which are in conformity for 10-oxodocetaxel as per their report (40). The types and percentage of impurities formed at different temperature of hydration and annealing are shown in Fig. 4 and Table III. As the temperature increases the percentage and number of impurities also increased. Therefore, the generation of DTX impurities are temperature and time dependent. However, with increase in temperature and annealing time the DTX loading also increases (Table III) and so a compromise in DTX loading will have to be practiced by optimizing the temperature and annealing time to reduce the impurity generation below the accepted range. The preparation of liposomes with phospholipids having Tg above body temperature such as DPPC (41°C) and HSPC (60°C) will enhance the *in vivo* circulation time and also reduce the *in vivo* drug leakage as compared to unsaturated phospholipids and phospholipids having Tg below body temperature. Hence, it is beneficial to use these saturated phospholipids to prepare liposomes even though use of these lipids demands the annealing temperature above 60°C (41). However, the use of degradation inhibitors such as organic and inorganic acids in DTX injection has shown to protect DTX from degradation at this temperature.

Based on results obtained in preliminary experiments, the cholesterol concentration (X1), hydration and annealing temperature (*X*2) and citric acid concentration (X3) were found to be major variables affecting % impurity formation (Y1) and % DTX loading (Y2). Hence, 3^3 full factorial design was applied to find the influence of these variable parameters (X1, *X*2, X3) on the response parameters (Y1 & Y2) and to optimize conditions for preparing DTX liposomes with maximum drug loading and No/minimum impurities. Liposomes were prepared adopting procedure discussed earlier using drug lipid ratio of 1:30 and the organic acid (citric acid) was added during film formation to avoid the chances of degradation of DTX during film formation. A total of 27 batches of DTX loaded liposomes were prepared and their details are given in Table I.

Results of factorial was first analysed based on the influence of variable parameter on Y1 response (% impurity formation) since the ideal formulation expected should contain no/minimal concentration of impurity formed by the degradation of DTX. Basically, increase in cholesterol and citric acid concentration decreased the % impurity formation while, temperature of hydration and annealing increased the impurity formation. All batches (CA-AL-1,10,19) prepared with 0.4 mg of CA and varied concentration of cholesterol at 75°C showed no impurity formation while many batches prepared at 80°C

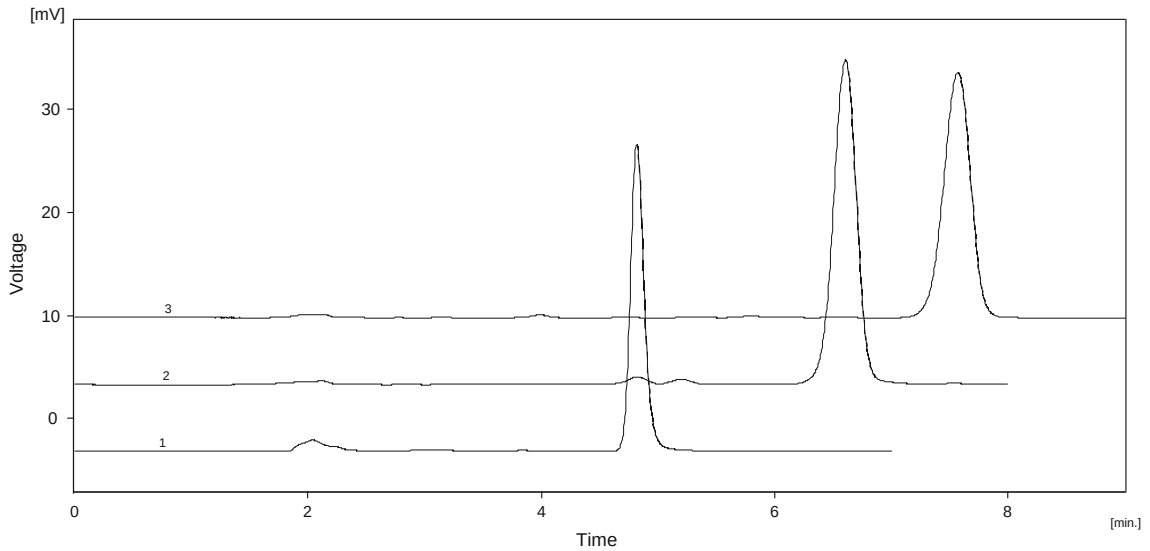

Fig. 3 Characteristic peaks of (1). DTX (RT:4.820±0.076 min; RRT:1) (2). EDTX (RT: 6.587±0.054 min; RRT:1.366) and (3). 10-oxo-7-epidocetaxel (RT: 7.573±0.042 min; RRT: 1.571).

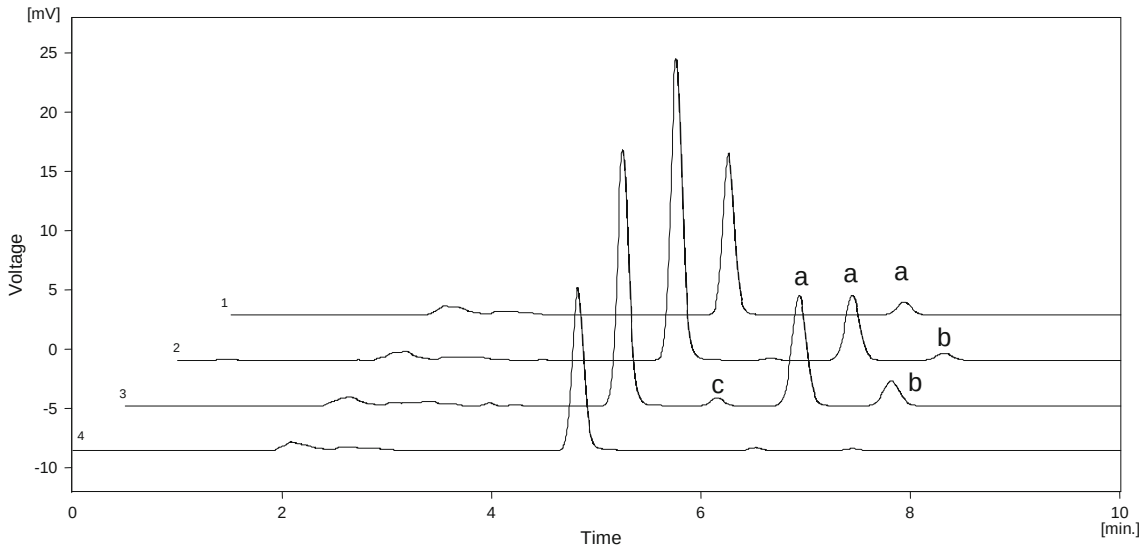

Fig. 4 Identification of types of impurities loaded in the liposomes prepared at different hydration temperature in the absence of organic acid. (1). liposomes prepared at 60°C (a: EDTX) (2). At 75°C (a: EDTX; b:10-oxo-7-epidocetaxel) (3). At 85°C (a:EDTX; b:10-oxo-7-epidocetaxel; c:10-oxodocetaxel) (4). liposomes prepared at 85°C in the presence of tartaric acid (0.4 mg).

and 85°C showed impurities. Similar observations were recorded with all batches (CA-AL-2,11,20) prepared with 1 mg CA at 75°C and batches prepared with 1 mg CA and 0.0522 mM of cholesterol at 80°C and 85°C. All batches prepared with 2 mg of CA irrespective of cholesterol concentration and temperature of hydration and annealing showed no impurity formation. These results conclude that (1) all batches prepared at 75°C irrespective of the concentration of cholesterol and CA and (2) batches prepared with highest concentration of cholesterol (0.0522 mM) with 1 mg/2 mg CA showed no impurity formation.

Analysis of the influence of variable parameters on % drug loading (Y_2 Response), showed positive response with increase in temperature of hydration and annealing (*X*2) but negative response with increase in the concentration of cholesterol (X1) and citric acid (X3). Reduced factorial design with 2 levels (Lowest and highest) of all the three factors (2*3) was conducted to study the magnitude of main and combined effect of the factors using the selected data from Table I. Details are given in Table II.

Main effects and combined effect of factors on the % drug loading calculated show temperature of hydration and annealing has maximum positive contribution (+49.27%) followed by remarkable negative contribution of CA concentration (−40.73%). Cholesterol contribution on the % drug loading is also negative but not very significant (−3.67). Combined effects of two factors are positive but negligible in case of cholesterol conc.: Temp. (X1.*X*2) and Temp.: CA conc, (*X*2.X3) while combined effect of cholesterol conc.: CA conc. (X1.X3) was quite higher (+4.73) in comparison to others. Combined effect of all the three factors (X1.*X*2.X3) is also negligible (−1.04). These results clearly indicate that the two negative factors, cholesterol and CA concentration contradicts positive effect of temperature of hydration and annealing on % drug loading. Hence, liposomal batch prepared with high temperature of hydration and annealing with low cholesterol and CA concentration could yield high % drug loading as evidenced by batch number CA-AL-7 (57.62 ± 1.92%).

However, high temperature of hydration and annealing increases DTX degradation as evidenced by batch number CA-AL-7 where the % EDTX estimated is maximum (1.786 ± 0.234%) among the 27 batches tested (Table I). Looking into batches of liposomes showing no formation of EDTX, it is

Table III Comparison of % Docetaxel and Impurities Loaded in Liposomes at Different Hydration Temperature in the Absence of Organic Acid

Temperature (°C)	% DTX	% 10-oxo docetaxel	% EDTX	% 10-oxo-7-epidocetaxel	% Total loading
60 ± 1	33.9 ± 3.4	–	2.96 ± 0.23	–	36.86 ± 3.6
75 ± 1	61.64 ± 2.6	–	14.78 ± 1.4	1.602 ± 0.3	78.022 ± 4.3
85 ± 1	63.54 ± 1.9	2.34 ± 0.47	25.66 ± 1.1	6.6 ± 0.5	95.1 ± 3.9

Values are Mean ± SD, $n = 3$. The docetaxel loading increased highly significantly at 75°C and 85°C as compared to 60°C (***$p < 0.0001$). Similarly, the % EDTX loading decreased highly significantly at 60°C as compared to 75°C and 85°C (***$p < 0.0001$)

interesting to note that those batches prepared at 75°C with (1) 0.0232 mM of cholesterol and varied concentrations of CA (CA-AL-1, 2 & 3); (2) 0.0348 mM cholesterol and varied concentrations of CA (CA-AL-10, 11 & 12) and (3) 0.0522 mM of cholesterol and varied concentrations of CA (CA-AL-19, 20 & 21) are included. However, it is also noted that, DTX degradation due to exposure to higher temperature of hydration and annealing was prevented by increasing the concentration of citric acid. For example from Table I, batches prepared with 2 mg CA (irrespective of cholesterol content & temperature of hydration and annealing) showed no evidence of DTX degradation. Similarly, increasing cholesterol content to 0.0522 mM protected DTX from degradation in batches prepared at 80°C with 1 mg and 2 mg CA (CA-AL-23 & 24) and at 85°C with 1 mg & 2 mg CA (CA-AL-26 & 27).

The liposome batch, CA-AL-25, prepared at 85°C of hydration & annealing with cholesterol (0.0522 mM) and citric acid (0.4 mg) showed evidence of DTX degradation (0.558 ± 0.079%) within the Indian pharmacopoeial limit (should be less than 1%) and significant drug loading (49.64 ± 3.12%) was optimized. Also, this batch comprises high cholesterol concentration so that we can expect decreased *in vivo* DTX leakage and enhanced *in vivo* liposomal circulation time.

The pH of formulation prepared with 0.4, 1 and 2 mg citric acid at all cholesterol and temperature levels was found to be 5.305 ± 0.184, 4.454 ± 0.102 and 3.832 ± 0.105, respectively. As CA concentration increases, the pH of final formulation decreases which then decreases the impurity generation even after heating the formulation at higher temperature (85°C) during hydration and annealing for a period of 1.5–2 h. Hence, we can conclude that the pH of formulation also plays a pivotal role in controlling the degradation of DTX and the DTX remain more stable at pH near 4.0 (marketed TXT having pH of 4.0 was adjusted with citric acid) than at higher pH. Optimized liposomal formulation described above has the pH of 5.1 (with 0.4 mg CA) with substantial drug loading and minimum EDTX.

At the optimized level of cholesterol (0.0522 mM), temperature (85°C), hydration and annealing time of 1.5–2 h and probe sonication time of 1 min we have tested the efficiency of other organic acids (ascorbic acid, maleic acid, and tartaric acid), as degradation inhibitor, at the optimized concentration of citric acid (0.4 mg). The effect of these acids on % DTX loading and % impurity generation was shown in Fig. 5 and Table IV. No significant change in the % DTX loading with these acids (except maleic acid) was observed as compared to citric acid. We observed very less amount of impurities in liposomes prepared with tartaric acid (0.144 ± 0.128%) as compared to citric acid (0.558 ± 0.079%), maleic acid (0.609 ± 0.0997%) and ascorbic acid (1.559 ± 0.666%). This decreased impurity generation is due to better control of liposomal pH by tartaric acid (pH 4.408 ± 0.11) as compared to other acids (Table IV). The decrease in pH of formulation was better correlated to pKa of these acids used in the formulation. The pKa of tartaric acid was less than the other acids used (Table IV), hence it controlled the pH better and thereby the degradation of DTX in liposomes.

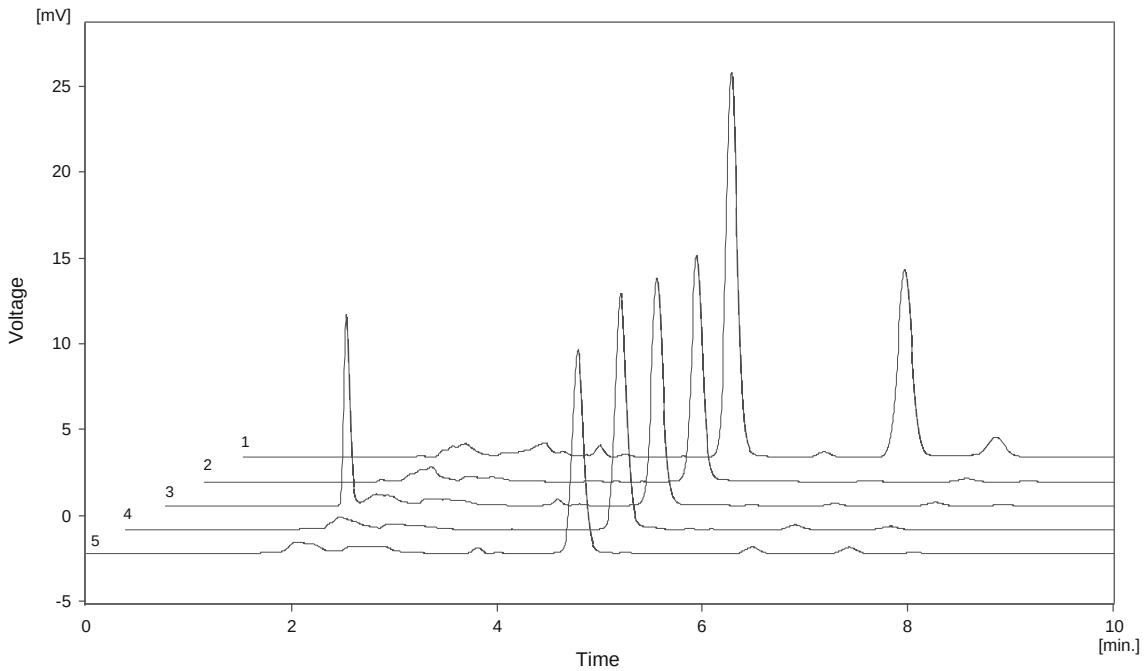

Fig. 5 Comparison of liposomal formulations containing Vitamin E (1) and organic acids as degradation inhibitors [2: Tartaric acid (0.4 mg); 3: Maleic acid (0.4 mg); 4: Citric acid (0.4 mg); and 5: Ascorbic acid (0.4 mg)].

Table IV Comparison of Vitamin E and Organic Acids as Degradation Inhibitors

Organic acid	pH values	% DTX loaded	% EDTX loaded
CA (pKa;3.12, 4.76 & 6.39)	5.305 ± 0.184	47.566 ± 2.59	0.558 ± 0.079
AA (pKa; 4.17 & 11.57)	5.43 ± 0.22	42.273 ± 5.13	1.559 ± 0.666
MA (pKa;3.4 & 5.2)	5.043 ± 0.121	30.486 ± 5.18	0.609 ± 0.0997
TA (pKa; 2.93 & 4.23)	4.408 ± 0.11	45.72 ± 4.43	0.144 ± 0.128
Vitamin E (as anti-oxidant)	6.71 ± 0.24	56.373 ± 2.10	27.113 ± 2.052

Values are Mean ± SD, $n = 3$

CA citric acid, *AA* ascorbic acid, MA maleic acid, TA tartaric acid

Significantly decreased docetaxel loading was observed with maleic acid as compared to citric acid (*$p < 0.05$). No significant difference in % EDTX loading was observed between citric acid and other acids but found significant increase in % EDTX loading with Vitamin E (***$p < 0.0001$) as compared to all other acids

Vitamin E is having an anti-oxidant property and might prevent oxidative degradation of DTX to form 10-oxodocetaxel and 10-oxo-7-epidocetaxel. Hence, we also tested vitamin E as degradation inhibitor at the same concentration of citric acid (0.4 mg). Our results reveal that vitamin E is unable to prevent oxidation reactions in our experimental conditions. The liposomal formulation prepared with vitamin E (pH 6.71 ± 0.24; which is similar to formulation containing no acids) shows all three possible impurities (10-oxodocetaxel, EDTX and 10-oxo-7-epidocetaxel) at our experimental conditions (Fig. 5). We used tartaric acid, as compared to citric acid and other acids, as degradation inhibitor in the liposomal formulation as it better control the impurity generation without significantly affecting the DTX loading. The final optimized CLs showed mean particle size of 111 ± 5 nm (PDI: 0.266 ± 0.101) and zeta potential of −40.2 ± 2.4 mV.

Preparation of PLs

The PLs were prepared using DSPE-mPEG$_{2000}$ at different mol% by pre-insertion technique and analysed for mean particle size, % DTX and impurity loading and pH. Upon increasing the DSPE-mPEG$_{2000}$ concentration we observed significant increase in DTX loading at 3 mol% (*$p < 0.05$) and 5 mol% (***$p < 0.0001$) as compared to CLs (CLs: 45.72 ± 4.59%; PLs-1 mol%: 50 ± 4.43%; PLs-3 mol%: 60 ± 2.43%; and PLs-5 mol%: 78.358 ± 2.863%). The 5 mol% PLs showed significantly increased DTX loading than 3 mol% PLs (*$p < 0.05$, $p = 0.0017$). This is might be due to increased total lipid content after inclusion of 5 mol% DSPE-mPEG$_{2000}$. Although the mean particle size remain unchanged at 1 and 3 mol% (110 ± 2 nm; PDI: 0.262 ± 0.0070), we observed increased mean particle size at 5 mol% (119 ± 6 nm; PDI: 0.228 ± 0.0403) (Fig. 6a) as compared to CLs (107 ± 6 nm; PDI: 0.275 ± 0.024). The impurity (EDTX) formation was increased with increase in DSPE-mPEG$_{2000}$ concentration (CLs: 0.144 ± 0.128%; PLs-1 mol%:0.18 ± 0.045%; PLs-3 mol%:0.32 ± 0.121%; and PLs-5 mol%:0.462 ± 0.062%). This increased impurity generation might be better correlate to the increased formulation pH with increase in DSPE-mPEG$_{2000}$ concentration (CLs: pH 4.4 ± 0.02; PLs-1 mol%: pH 4.5 ± 0.043; PLs-3 mol%: pH 4.7 ± 0.022; and PLs-5 mol%: pH 5 ± 0.11). The zeta potential of prepared PLs was found to be increased at all DSPE-mPEG$_{2000}$ concentrations (−56 ± 2 mV) as compared to CLs (−42.5 ± 1.7 mV). The zeta potential of PLs was more negative than that of CLs due to the negatively charged phosphate group of DSPE-mPEG$_{2000}$, which is also in accordance with the earlier reports (28,42). According to Yang *et al.* (28) report the paclitaxel loaded CLs, composed of S_{100}PC/Cholesterol (90:10, molar ratio), showed zeta potential of −1.23 ± 0.64 mV whereas the PLs, composed of S_{100}PC/Cholesterol/DSPE-mPEG$_{2000}$ (90:10:5, molar ratio), showed zeta potential of −16.25 ± 2.15 mV.

We determined the role combination of phospholipids (HSPC:DPPC), instead of individual phospholipids, in DTX loading by preparing PLs (5 mol%) containing HSPC and DPPC both and individually. We observed significant increase in DTX loading with PLs composed of both HSPC and DPPC (78.358 ± 2.863%) as compared to PLs composed of only HSPC (66.73 ± 4.23%) (*$p < 0.05$, $p = 0.024$) or only DPPC (57.92 ± 3.82%) (*$p < 0.05$, $p = 0.0014$). About 11% and 20% increase in DTX loading was observed as compared to HSPC PLs and DPPC PLs, respectively. Therefore it concludes that the use of combination of phospholipid (rather than single phospholipid) would enhance the hydrophobic drug loading in the membrane bilayer. The slight increase in % impurity was observed with PLs composed of only HSPC (0.641 ± 0.162%) or only DPPC (about 0.558 ± 0.0921%) as compared to PLs composed of both HSPC and DPPC (0.462 ± 0.062%). This is might be due to altered rigidity of bilayer membrane composed of both phospholipids as compared to membrane composed of individual phospholipids. No significant change in mean particle size was observed.

Transmission Electron Microscope Analysis

The TEM image of 5 mol% PLs (Fig. 6b) clearly reveals that the prepared liposomes are of spherical in nature. Also, the TEM image depicts liposomes are of slightly poly-disperse in nature as shown by Malvern Zeta Seizer analysis (polydispersity index (PDI): 0.228 ± 0.0403).

Differential Scanning Calorimetry Analysis

The sensitivity of glass transition is directly proportional to sample heating rate and sample mass (43–45). Therefore, in

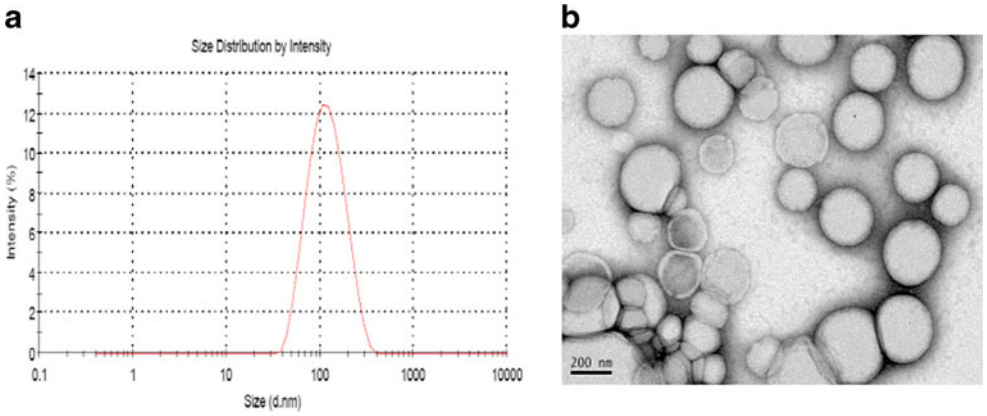

Fig. 6 The average mean particle size of 5 mol% PEGylated liposomes (119 ± 6 nm) determined by (**a**) Malvern Zetasizer Nano and (**b**) TEM Image showing spherical morphology of 5 mol% PEGylated liposomes.

the present study, the DSC analysis was performed at high heating rate of 100°C per minute in order to detect even small amount drug that undergone polymorphic change. The DSC thermogram of plain DTX (Fig. 7a) showed broad peak, corresponding to DTX, in the range of 180–188°C. The DSC thermogram of plain DTX also shows a peak at 115.8°C corresponds to loss of residual water. In the DSC thermogram of physical mixture (Fig. 7b) we observed a similar broad peak, corresponding to DTX, at 187.7°C whereas the same melting peak was absent in the DSC thermogram of DTX loaded PLs (Fig. 7c). This indicates that the DTX is present in molecular state after being entrapped into the liposomal bilayer. The DSC thermogram of physical mixture also showed two endothermic peaks, corresponding to lipid mixture, one at 81.4°C and second at 130.9°C. The similar peaks were also observed in the DSC thermogram of DTX loaded PLs (one at 66°C and second at 137°C).

In Vitro Serum Protein Adsorption Study

Bonte and Juliano (46) demonstrated that liposomes rapidly bind a complex profile of plasma proteins *in vitro* upon exposure to human plasma or serum. The presence of PEG on liposomal surface attracts a water shell, resulting in reduced adsorption of plasma proteins such as opsonins and, as a consequence, impaired recognition of liposomes by the cells of the mononuclear phagocyte system (MPS) following intravenous administration. This, in turn, is believed to lead to extended blood circulation times of these liposomes (26). The *in vitro* determinations, being simpler and allowing for greater recoveries of liposomes should therefore be a useful assay for predicting the *in vivo* clearance behaviour of liposome of novel compositions (27).

The recovered liposomes (Supplementary Material STI) were first analysed for mean particle size, zeta potential retention as serum protein adsorption can alter both these characters. The CLs, PLs-1 mol%, 3 mol% and 5 mol% showed mean particle size of 107 ± 6 nm (PDI: 0.275 ± 0.024), 108 ± 2 nm (PDI: 0.262 ± 0.0070), 108 ± 2 nm (PDI: 0.262 ± 0.0070) and 119 ± 6 nm (PDI: 0.228 ± 0.0403), respectively before incubation with serum. After recovered from the serum, they showed mean particle size of 139 ± 5 nm (PDI: 0.2955 ± 0.042), 116 ± 1 nm (PDI: 0.211 ± 0.009), 127 ± 5 nm (PDI: 0.241 ± 0.011) and 122 ± 3 nm (PDI: 0.241 ± 0.017), respectively. About 30 nm increase in mean particle size was observed with CLs (**$p < 0.001$) as compared to 1 mol% (about 8 nm), 3 mol% (about 14 nm, *$p < 0.05$, $p = 0.0094$) and 5 mol% (negligible change) PLs after recovered from the serum. The 5 mol% PLs better retained their mean particle size and this is might be due to decreased association of serum proteins as compared to 1 mol% PLs, 3 mol% PLs and CLs. The zeta potential of recovered CLs (decreased from −42.5 ± 1.7 mV to −16.5 ± 4.9 mV) and PLs (decreased from −55.5 ± 2.4 mV to −13.05 ± 0.21 mV) was found significantly decreased.

The liposome associated proteins were efficiently extracted and delipidated as described in the method (Supplementary Material). This delipidation step is required because lipids interfere with most protein assays (47). The CLs (P_B: 494.417 ± 22 μg/μM of total phospholipid recovered) showed significantly high serum protein binding (P_B) as compared to PLs with 1 mol% (325.899 ± 15 μg/μM), 3 mol% (297.194 ± 25 μg/μM) and 5 mol% (242.085 ± 15 μg/μM) of DSPE-mPEG$_{2000}$ (***$p < 0.0001$) (Supplementary Material STII and SF1). The PLs with 5 mol% DSPE-mPEG$_{2000}$ showed significantly less amount of associated serum proteins as compared to 3 mol% DSPE-mPEG$_{2000}$ (*$p < 0.05$) indicating the possibilities of low level of opsonisation and phagocytosis. Therefore, we can expect enhanced *in vivo* circulation time with 5 mol% PLs as compared to CLs and other PLs (1 mol% and 3 mol%).

In Vitro Drug Release Study

The 0.1% (w/v) tween-80 was added in the release medium (PBS, pH 7.4) to maintain sink condition during the release study. The *in vitro* drug release from TXT, PLs composed of HSPC alone, DPPC alone and both HSPC and DPPC (1:1) was studied at room temperature and results are presented in Fig. 8 (also see Supplementary Material STIII). The drug

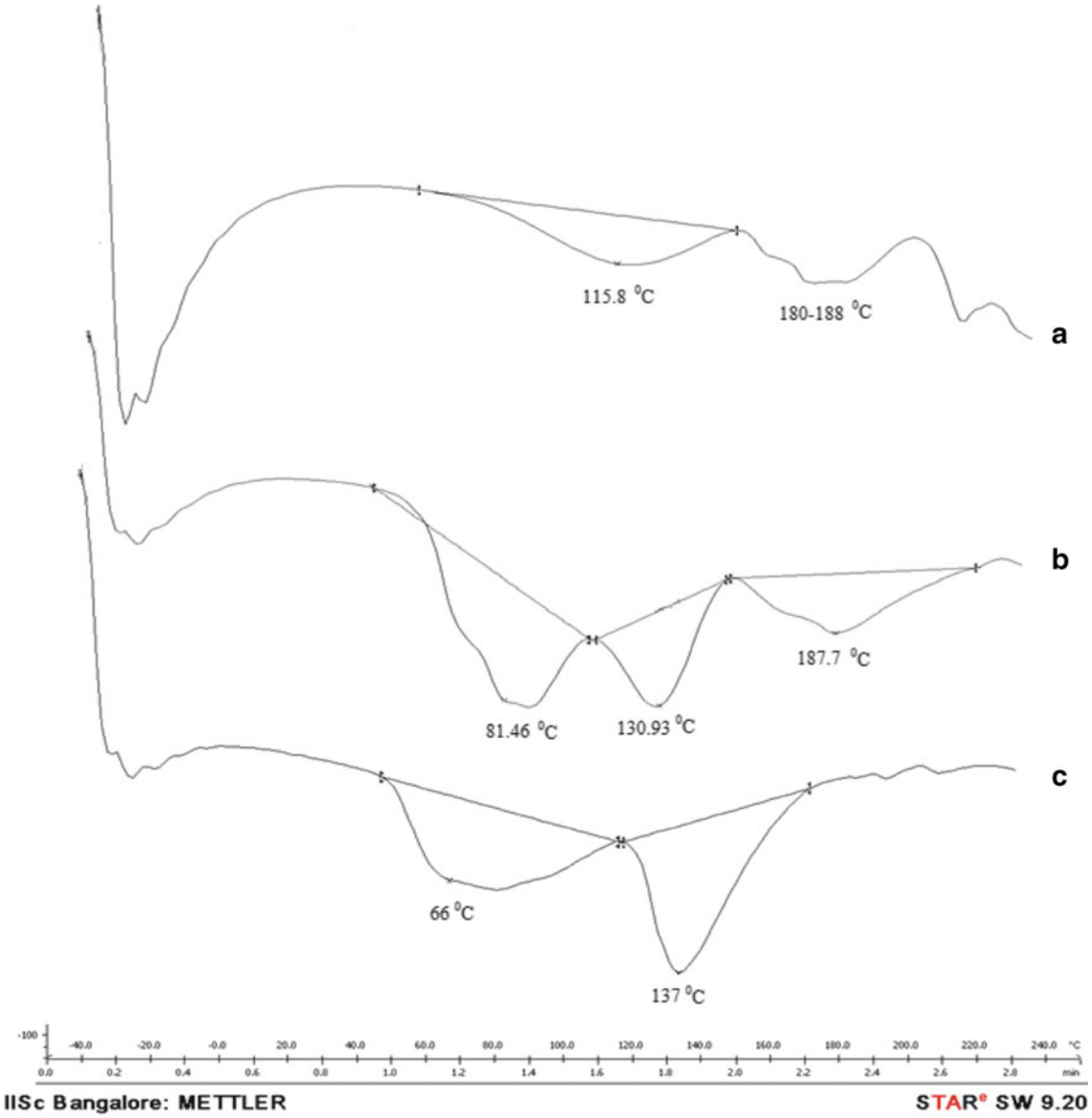

Fig. 7 DSC thermogram of (**a**) plain docetaxel, (**b**) physical mixture and (**c**) freeze dried docetaxel loaded PEGylated liposomes.

release was better controlled from all PLs as compared to marketed TXT (49.957±4.223%) during 48 h of release study. The PLs composed of both HSPC and DPPC showed better control of DTX release (19.898±0.507%) as compared to PLs composed of HSPC alone (31.976±1.503%) or DPPC alone (26.454±0.341%) after 48 h of release study. This more controlled release of DTX from PLs, composed of both HSPC and DPPC, is might be due to altered membrane stability as compared to liposomes composed of individual phospholipids and thus exhibiting depot effect. From the above results it is very clear that the PLs, composed of both HSPC and DPPC, would show very slow release of DTX in the blood circulation, targets more DTX to tumor tissues and therefore meets the requirements for an effective drug delivery system.

The epimerization of released DTX into EDTX in the release medium was observed in our study. More the DTX released from formulation (TXT), more will be the formation of EDTX in release medium (14.06±2.09% after 48 h of study) (Table V). The PLs composed of both HSPC and DPPC showed very less drug release after 48 h study therefore less conversion of DTX into EDTX (4.37±0.64%) in the release medium. Therefore, the PLs would better control the DTX release in blood and reaches the maximum amount of active DTX, instead of EDTX which helps tumor to develop resistance to DTX chemotherapy, to the tumor tissue. Significant amount of impurities remain unreleased from TXT formulation as compared to HSPC:DPPC PEGylated liposomal formulation (Table V) after 48 h of incubation in PBS (pH 7.4). Therefore, we can expect better

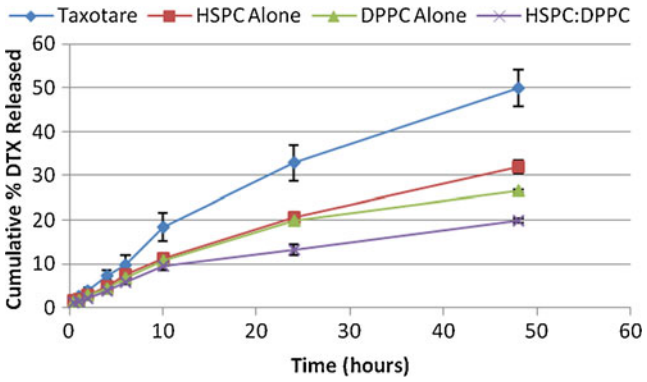

Fig. 8 Comparison of cumulative % DTX released from different formulations. Values are Mean±SD, $n=3$. The % docetaxel released from all liposomes was found significantly less than TXT (***$p<0.0001$) after 48 h of release study. The % docetaxel released from HSPC:DPPC liposomes was found significantly less than HSPC liposomes (*$p<0.05$, $p=0.001$) and DPPC liposomes (*$p<0.05$, $p=0.045$) after 48 h of release study.

control of metabolism of loaded DTX into EDTX, in blood circulation, with PLs composed of both HSPC and DPPC than marketed TXT. Thus, DTX loaded liposomal chemotherapy is necessary for better treatment of solid tumors (and other cancers) with less chance of tumor resistance to chemotherapy from DTX impurity (EDTX).

The obtained DTX release pattern and impurity formation from TXT and liposomal formulations is at room temperature. However, the DTX release profile and impurity profile can alter at physiological temperature (37°C).

In Vitro Cytotoxicity Study

The liposomal formulations prevent the toxic effect of drug applied at high concentration and thus can increase the maximum tolerance dose (MTD). Such a high concentration of drug instantly exposed in blood is presumed to be toxic not only for cancer cells but also for normal cells since it has exceeded the suggested maximum tolerable level of DTX (2700 ng/mL) (48). All formulations resulted in concentration and time dependent inhibition of proliferation of A549 (Supplementary Material SF2) and B16F10 cells (Supplementary Material SF3). It is clear from the results that the PLs demonstrated higher cytotoxicity than TXT formulation at the same drug concentration and exposure time, which means that for the same therapeutic effect the drug needed for PLs could be much less than that for TXT formulation. Therefore, the development of PLs thus can enhance the therapeutic effect as well as increase the MTD of DTX.

A quantitative evaluation of *in vitro* therapeutic effect of a dosage form is IC_{50}, which is defined as the drug concentration needed to kill 50% of incubated cells in a designated time period (Table VI). The IC_{50} value after 24 h treatment of A549 cells was found to be higher for TXT (25±4.082 nM) than PLs (0.5±0.01 nM). Therefore PLs showed high

Table V Formation of Docetaxel Impurities in Release Medium of Different Formulations

Time (hr)	TXT		PEGylated Composed of					
			HSPC		DPPC		HSPC: DPPC	
	% EDTX	%10-Oxo	% EDTX	%10-Oxo	% EDTX	%10-Oxo	% EDTX	%10-Oxo
10	3.16±0.57	–	0.80±0.10	–	0.793±0.09	–	0.39±0.02	–
24	6.89±1.70	–	3.08±0.22	–	3.43±0.072	0.28±0.084	1.96±0.11	–
48	14.06±2.09	1.95±0.22	7.31±0.39	0.70±0.095	7.85±0.77	0.71±0.079	4.37±0.64	0.27±0.17
Impurities remain unreleased from formulations after 48 h of incubation with PBS (pH 7.4)								
	11.34±3.29	1.254±0.12	4.66±0.18	0.25±0.05	2.81±0.32	0.16±0.11	1.86±0.24	0.13±0.03

Values are Mean±SD, $n=3$. 10-Oxo: 10-oxo-7-epidocetaxel. The % EDTX formed in the release medium was found significantly high with TXT as compared to HSPC liposomes (**$p<0.001$), DPPC liposomes (*$p<0.05$, $p=0.00116$) and HSPC: DPPC liposomes (***$p<0.0001$) after 48 h of release study. The % EDTX formed in the release medium was found significantly less with HSPC:DPPC liposome as compared to DPPC liposomes (*$p<0.05$) after 48 h of release study. The % EDTX formed and remain unreleased from TXT after 48 h of release study was found significantly high as compared to HSPC liposomes (*$p<0.05$), DPPC liposomes (*$p<0.05$) and HSPC:DPPC liposomes (**$p<0.001$)

Table VI The Comparison of IC_{50} Values (nM) of TXT, CLs and PLs Against A549 and B16F10 Cells at Different Time Points

Drug	A549			B16F10		
	24 h	48 h	72 h	24 h	48 h	72 h
TXT	25 ± 4.082	0.0051 ± 0.0017	0.005 ± 0.001	ND	0.425 ± 0.170	0.0512 ± 0.013
CLs	150 ± 10	1.51 ± 0.18	0.254 ± 0.021	90.67 ± 11.67	60.739 ± 5.769	10.639 ± 2.481
PLs	0.5 ± 0.01	0.0064 ± 0.001	0.004 ± 0.001	4000 ± 807	0.009 ± 0.001	0.005 ± 0.0012

Values are Mean ± SD, $n = 3$

ND not detectable

therapeutic effectiveness (50 times more) as compared to TXT after 24 h study. After 48 and 72 h of treatment, the IC_{50} values were found decreased as compared to 24 h treatment and were observed almost same for both TXT and PLs. This indicates that both TXT and PLs are almost equally effective after 48 and 72 h treatment. The CLs showed higher IC_{50} values than TXT at all time points indicating less effective than TXT. This less cytotoxicity can be attributed to low amount of loaded DTX in a high proportion of lipids leading to more time to diffuse and come in contact with the cells.

In case of B16F10 cells the IC_{50} values were shifted to higher values after 24 h of treatment as compared to A549 cells indicating B16F10 melanoma cells are less sensitive than A549 cells. After 48 and 72 h of treatment the IC_{50} values of PLs almost matches with the IC_{50} values against A549 cells. The PLs showed lower IC_{50} values than TXT at all time points against B16F10 cells indicating better therapeutic efficacy of PLs than the marketed TXT. The CLs again showed higher IC_{50} values than TXT after 48 and 72 h time points indicating less effective than TXT.

The PLs showed lower IC_{50} values (more effective) as compared to TXT against both the cell lines. These results are in accordance with previous studies that the cytotoxicity of drug-loaded lipid based nanoparticles was higher than that of free drugs (49,50). In many solid lipid nanoparticle (SLN) formulations less than half of the loaded drug is released during the cell cytotoxicity test, but these formulations are sometimes more cytotoxic to the cancer cells than the corresponding free drug. In other words, the cytotoxic compounds that remain unreleased and associated with the lipid based nanoparticles also appear effective (51,52). Possible mechanism underlying the enhanced efficacy of DTX loaded nano structured lipid carriers (NLC) against cancer cells is that the lipid nanoparticles carry the drug into cancer cells by endocytosis and enhance intracellular drug accumulation by nanoparticle uptake (51). Therefore, the increased cytotoxicity of DTX loaded PLs compared to marketed TXT in our study can be better correlate to the reasons discussed above.

The cytotoxicity of blank PLs at different lipid concentrations was determined. The total lipid concentration used was similar to CLs used to study the cytotoxicity of loaded DTX (1.5504 μM, 155.04 nM, 15.504 nM, 1.5504 nM, 0.15504 nM, 0.015504 nM and 0.00155 nM). The blank PLs had no effect on A549 and B16F10 cell growth at total lipid concentration of 15.504 nM at all time points. Indeed, the differences in viability observed in cells that were incubated with blank PLs and the non-treated cells were negligible. At higher concentrations of blank PLs (155.04 nM and 1.5504 μM), we observed both concentration and time dependent cytotoxicity against both the cell lines. Therefore, in our present study, the cytotoxicity of DTX loaded PLs (54 nM of total lipid was used at 10 μM DTX concentration) was carried out at total lipid concentration that is about 3 times less than the toxic level (155.04 nM) as compared to CLs. Therefore, the % viability of A549 and B16F10 observed at 1 μM and 10 μM of CLs loaded with DTX is because of both lipid and DTX, as lipids at this level also cause cytotoxicity.

In Vitro Cell Uptake Study

The A549, B16F10 and K9 cell uptake of 6-coumarin loaded CLs and PLs, equivalent to 25 μg and 50 μg of total lipid, was studied using flow cytometer (Supplementary Material SF4, SF5 and SF6). The % relative mean fluorescence intensity (% RMFI) of these cell lines, after uptake of CLs and PLs, was determined by considering the MFI of cells treated with CLs as 100% uptake/100% RMFI. The % RMFI of A549 cells was found to be 59.1 ± 1.18%, 56.4 ± 1.64%, 61.3 ± 2.38% and 54.6 ± 1.22% after incubation with 25 μg of PLs for 30 min and 60 min, 50 μg of PLs for 30 min and 60 min, respectively. Similarly, the % RMFI of B16F10 cells was found to be 46.3 ± 4.77%, 54.4 ± 5.13%, 50.46 ± 2.35% and 50.1 ± 5.18% after incubation with 25 μg of PLs for 30 min and 60 min, 50 μg of PLs for 30 min and 60 min, respectively. The uptake of CLs (non-PEGylated) was found very rapid against both the cell lines tested and also, it is very clear from the results obtained that the uptake of PLs was decreased highly significantly as compared to CLs against both the cell lines and at all concentrations and time points

tested (***$p<0.0001$). This result confirms the presence of hydrophilic PEG barrier on PLs and that prevented the rapid uptake of PLs by these cell lines.

The conventional liposomes are rapidly taken up by liver and spleen before reaching the target tissue (53). Hence, we tested the uptake of prepared CLs, PLs against rat normal liver cell K9 and from these *in vitro* results we can predict the *in vivo* status of our prepared liposomes during preclinical study using mice models. The % RMFI of K9 cells was found to be 57.3±5.28%, 60.7±4.55%, 63.9±5.93% and 57.8±3.32% after incubation with 25 µg of PLs for 30 min and 60 min, 50 µg of PLs for 30 min and 60 min, respectively. The hydrophilic PEG barrier significantly decreased the K9 cell uptake of PLs, as compared to CLs (***$p<0.0001$), in a similar passion to A549 and B16F10 cells. The about 40% decrease in % RMFI of K9 cells treated with PLs, as compared to CLs (100%), was observed. Therefore we can expect increased *in vivo* circulation time and increased passive accumulation of PLs at tumor tissue as compared to CLs.

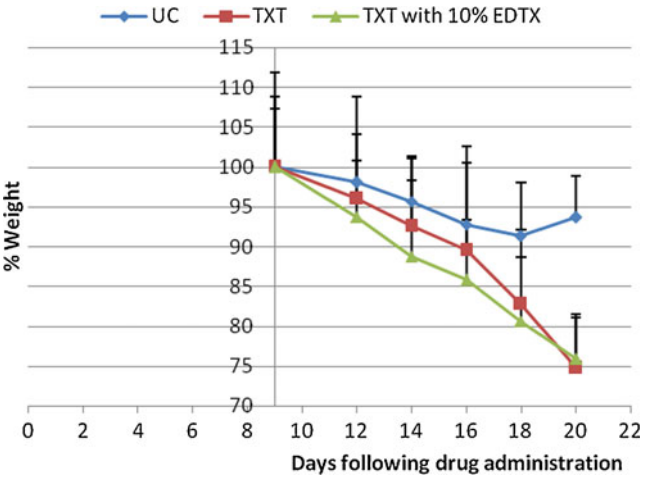

Fig. 9 The % group weights after administration of TXT alone and TXT with 10% EDTX separately. The TXT ($p<0.05$, $p=0.034$) and TXT containing 10% EDTX ($p<0.05$, $p=0.048$) treated groups showed significant weight loss as compared to untreated control group at the end of the experiment (Day-20). UC: Untreated Control.

Acute Toxicity Study

The *in vivo* B16F10 experimental metastasis model was used to compare the single *i.v.* dose acute toxicity of TXT for injection alone and with 10% EDTX (35,36). The weight measurement of mice was performed during the experiment to evaluate the systemic toxicity of TXT for injection and TXT containing 10% EDTX (Table VII and Fig. 9). At the end of experiment (Day-20), a significant weight loss was observed with TXT ($p<0.05$, $p=0.034$) and TXT containing 10% EDTX ($p<0.05$, $p=0.048$) treated groups as compared to untreated control group.

The control group showed normal and consistent mean group weight loss with time after PBS administration. The TXT injected group showed higher rate of weight loss as compared to PBS treated group. In comparison to control group the TXT treated group showed about 2.09, 1.73, 1.44, 1.98 and 4.05 fold increase in mean group weight loss at day 12, 14, 16, 18 and 20 of experiment, respectively. The inclusion of 10% EDTX in TXT injection resulted in 1.56, 1.5, 1.35 and 1.12 fold increased mean group weight loss as compared to TXT containing no EDTX at day 12, 14, 16 and 18 of experiment, respectively. This extra loss of body weight indicates the more systemic toxicity of EDTX as compared to DTX. Czejka *et al.* (4) reported the quantification of EDTX in blood and urine of chemotherapy patients (TXT). In 8 of 12 patients, EDTX was quantified in plasma at the end of infusion (concentrations ranging from 0.05 µg/mL to 0.54 µg/mL). In urine, EDTX has been found in 7 of 12 patients (ranged from 3.21 µg/mL to 66.37 µg/mL). These results indicate that there is significant amount of EDTX formation *in vivo* after TXT infusion (about 14% of injected dose) (4,54). Therefore, from our toxicity results, we can conclude that the total systemic toxicity that occurs during the chemotherapy with TXT is because of both DTX and EDTX that is formed *in vivo* after TXT infusion. Our *in vivo* toxicity study revealed higher systemic toxicity after inclusion of 10% EDTX to TXT as compared to TXT alone which clearly indicate that the prevention of DTX conversion into EDTX *in vivo* can enhance the therapeutic effectiveness of TXT with decreased systemic toxicity. Also, controlling the formation of EDTX *in vivo* will reduce the chance of tumor resistance to chemotherapy including DTX (6,7). As per our results the DTX remains stable at acidic pH, therefore it could also remain stable in the acidic tumor environment (55,56) than that in circulation (blood pH). Thus, an alternative drug delivery system (like liposome) that controls the release of DTX in circulation (we can expect better *in vivo* controlled release from our *in vitro* drug release data) and passively targets the DTX to tumor tissue is very much in need. In addition, the system should release the DTX at acidic tumor tissue, which in turn reduces/prevents the EDTX formation, for better

Table VII The % Group Weights After Administration of TXT Alone and TXT with 10% EDTX Separately

Day	Untreated control	TXT	TXT with 10% EDTX
9[a]	100±7.25	100±11.91	100±8.79
12	98.09±6.03	96.01±12.7	93.75±7.09
14	95.69±5.36	92.53±8.84	88.8±9.53
16	92.76±9.88	89.55±11.05	85.8±7.5
18	91.3±6.7	82.73±9.33	80.63±7.98
20	93.66±5.1	74.76±6.35	75.9±5.5

[a] Day when the drug was administered, Values are Mean±SD

Fig. 10 Appearance of lungs after resection from C57BL/6 mice injected intravenously with highly metastatic B16F10 melanoma cells: (**a**) untreated control (PBS); treatment with a single dose of (**b**) TXT 40 mg/kg; and (**c**) TXT containing 10% EDTX.

therapeutic effect with minimum systemic cytotoxicity and decreased chance of tumor resistance to chemotherapy.

On day-19 of experiment, one animal from the control group and one from TXT with 10% EDTX treated group

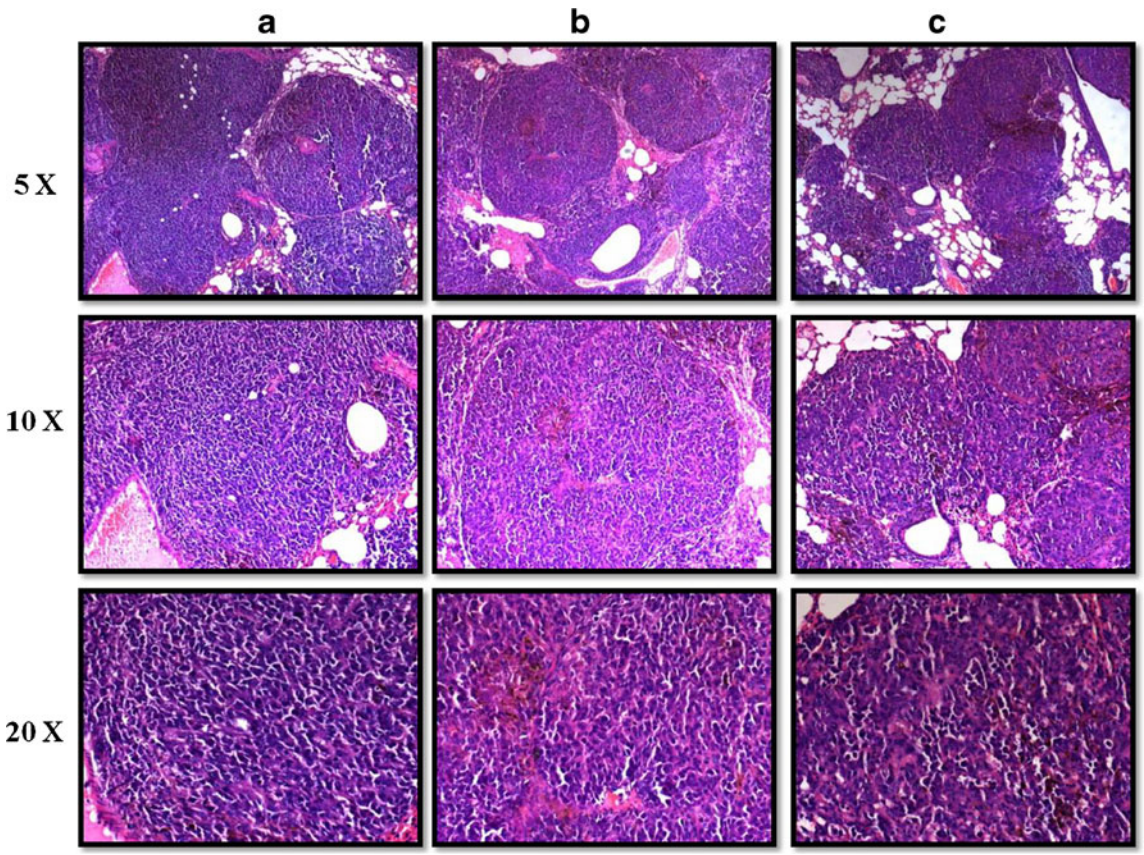

Fig. 11 HE staining of the lungs from C57BL/6 mice injected with B16F10 cells: (**a**) untreated control (PBS); treatment with a single dose of (**b**) TXT 40 mg/kg; and (**c**) TXT containing 10% EDTX. Images were captured at X5, X10 and X20 magnifications.

was found dead. On day-20 the animals were sacrificed, lungs were excised and photographed (Fig. 10). The lungs of control group, TXT treated group and TXT with 10% EDTX treated group were found completely black and metastatic nodules were remained uncountable (Fig. 10a–c). Therefore no therapeutic effect (decreased colony formation) was observed with TXT and TXT with 10% EDTX treated groups at 40 mg/kg single dose as compared to untreated control group. The hematoxylin and eosin (HE) stained lung section images of control (Fig. 11a), TXT alone (Fig. 11b) and TXT with 10% EDX treated groups (Fig. 11c) showed more number of large and fused nodules. The images are shown at different magnifications *viz.* X5, X10 and X20, respectively. A generalised dissemination of tumor cells is clearly visible across the lung parenchyma. Varying types of tumor islands are present throughout that have coalesced with each other. Further, the growing tumors had demolished the muscular coat of bronchioles as well as the vasculature in all three groups under investigation.

CONCLUSION

The docetaxel loaded PEGylated liposomes showed better control over the formation of 7-epimer in the release medium as compared to TXT. Therefore the prepared PEGylated liposomes, as compared to TXT, could show less *in vivo* conversion of loaded docetaxel into 7-epimer, more passive targeting of loaded docetaxel to tumor tissues and decreased 7-epimer induced systemic toxicity and tumor resistance to chemotherapy. Therefore, the further *in vivo* studies are needed to ascertain these facts. Also, the PEGylated liposomes could show decreased *in vivo* liver uptake, enhanced circulation half life, more passive accumulation in tumor tissues and enhanced therapeutic effect of loaded docetaxel. The *in vivo* pharmacokinetic and tumor distribution studies are further needed to ascertain these facts.

Statistical Analysis

All data were expressed in the form of mean ± standard deviation. Statistical analysis was performed using SPSS v.16 software. One Way ANOVA was done using Bonferroni test. In all cases, $p < 0.05$ was considered statistically significant.

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