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Research Article

A Novel Folate-Targeted Nanoliposomal System of Doxorubicin for Cancer Targeting

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Abstract. Targeted drug delivery systems for cancer improves anti-tumor efficacy and reduces systemic toxicity by restricting availability of cytotoxic drugs within tumors. Targeting moieties, such as natural ligands (folic acid, transferrin, and biotin) which are overexpressed on tumors, have been used to enhance liposome-encapsulated drug accumulation within tumors and resulted in better control. In this report, we explored the scope of targeting ligand folic acid, which is incorporated in liposome systems using folic acid-modified cholesterol (CPF), enabled highly selective tumor-targeted delivery of liposome-encapsulated doxorubicin and resulted in increased cytotoxicity within tumors. Folate-tagged poloxamer-coated liposomes (FDL) were found to have significantly higher cellular uptake than conventional poloxamer-coated liposomes (DL), as confirmed by fluorometric analysis in B16F10 melanoma cells. Biodistribution study of the radiolabeled liposomal system indicated the significantly higher tumor uptake of FDL as compared to DL. Anti-tumor activity of FDL against murine B16F10 melanoma tumor-bearing mice revealed that FDL inhibited tumor growth more efficiently than the DL. Taken together, the results demonstrated the significant potential of the folate-conjugated nanoliposomal system for drug delivery to tumors.

KEY WORDS: bioconjugation; cancer targeting; doxorubicin; folic acid; liposomes.

INTRODUCTION

Chemotherapy is the most important and established mode of treatment for cancer. However, the major problem with chemotherapeutic agents is their lack of specificity and selectivity (1–3). In recent times, a lot of efforts have been directed to improve the specificity of anticancer agents, based on the morphological and physiological differences between malignant and normal tissues (4). Malignant tissue has a leaky vasculature, which allows the cells to permit rapid movement of micro and macromolecules across them; this phenomenon is called enhanced permeability and retention (EPR) effect. Passive targeting is achieved using EPR effect whereby macromolecules and micro/nano particulates (typically liposomes and nanoparticles) tend to accumulate in tumor tissue much more than they do in normal tissues.

The rapid growing tumor cells require various nutrients and vitamins in large quantities for rapid development, and hence, they overexpress many tumor-specific receptors (eg., folate, biotin, transferrin, EGF) (3). These targets can be used to deliver cytotoxic agents into tumors. Vitamins like folic acid are required for the growth of cells as they are used in various metabolic cycles to synthesize different amino acids. An active targeting can thus be achieved by attaching the ligand for overexpressed receptors on the surface of delivery systems for enhanced uptake by tumor cells.

In the present research, active targeting approach using folic acid as the ligand has been explored. Several studies have shown that more than 80–90% of ovarian tumors overexpress folic acid receptors (FR) (5,6). In addition, other gynecological cancers as well as pediatric ependymal brain tumors, mesothelioma, and breast, colon, renal, lung tumors, and acute myelogenous leukemia also overexpress FR (7–9). The total number of tumors that express FR is very large; therefore, FR-targeted strategies could have significant beneficial impact on treatment of several types of cancer (10). FR is a useful target for tumor-specific drug delivery, because it is upregulated in many human cancer, and access to the folate receptor in normal tissues that express it can be low due to its location on the apical (externally facing) membrane of polarized epithelia. Moreover, folate receptor density appears to augment as the stage/grade of the cancer worsens. Hence, complicated therapy of cancer may be treated by a more straightforward approach of targeting using folate-linked therapeutics (10).

The lipid bilayer structure of liposomes is easily amenable to surface modifications by conjugating with various ligands like antibodies, antibody fragments, vitamins, glycoproteins,

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peptides (RGD sequences), and oligonucleotide aptamers, and active targeting to cancer cells is possible with such ligand-tagged liposomes (11).

Also, liposomal delivery systems are reported to be taken up by the reticuloendothelial system (RES). Surface modification of liposomes by coating them with hydrophilic polymers like PEGs and polaxamers (stealth liposomes) is one of the methods to avoid RES uptake (12).

Several attempts to develop folic acid-based liposomal delivery systems are reported, which have revealed enhanced uptake of folate-tagged delivery systems as compared to untagged delivery systems in *in vitro* cell uptake studies (10). Initially, PEG tagged with folic acid (folic-PEG) was linked to phosphatidylamine (PE) to yield ligand-tagged lipid (folate–PEG-PE), which was then incorporated into the liposomal bilayer. Apart from PE, folic acid-linked distearoyl phosphatidylethanolamine (DSPE) has also been explored (13). Cholesterol is used in the preparation of liposomes to provide rigidity to the bilayer membrane. Attempts are also reported to link folic acid to cholesterol such as folate–PEG-cholesterol and folate–PEG-cholesteryl hemisuccinate (13,14). Studies with PEG as linker to attach folic acid with polylactic acid are also reported (15).

Biodistribution studies of radiolabeled folate-derivatized liposomes (folate–PEG-chol) has been compared with that of nontargeted liposomes in a murine tumor model, where in both targeted and non targeted liposomes have shown the same level of enhanced uptake in tumor (13,16). All studies report, conjugation of folic acid to lipid or polymer using PEG as a linker. The present study aimed at the exploration of a small linker p-aminobenzylamine for conjugation of folic acid with cholesterol and then the subsequent incorporation in liposomes. This conjugation approach with p-aminobenzylamine provides simplicity and versatility and can be applied to different targeting ligands.

The objective of present investigation was to develop and evaluate the potential of folate-tagged liposomal delivery systems for active targeting to cancer cells using doxorubicin (DOX) as model anticancer agent.

MATERIALS AND METHODS

Cholesterol chloroformate, folic acid, and cholesterol were purchased from Sigma Aldrich (Mumbai, India); DOX was a gift from Naprod Life Sciences Ltd. (Mumbai, India); dimethyl sulfoxide (DMSO), dicyclohexyl carbodiimide (DCC) n-hydroxy succinamide (NHS), diethyl ether, hexane, ethyl acetate, butanol, glacial acetic acid, and sodium dodecyl sulfate solution (SDS) were purchased from S.D. Fine Chem Ltd. (Mumbai, India). Trehalose (Hayashibara, Japan) was supplied as gift sample by Gangwal Chemicals Pvt. Ltd (Mumbai, India). Folate-free DMEM medium, trypsin EDTA, and fetal bovine serum (FBS) were obtained from Himedia Ltd. (Mumbai, India). All other chemicals and reagents used were of analytical grade. All the animal studies were approved by independent ethics committee of Bhabha Atomic Research Centre, Mumbai, India. All the procedures involved in the animal studies were performed in accordance with the recommendations of NIH guidelines for the proper use and care of laboratory animals.

Cell Lines and Animals

A549 human lung cancer cell line and B16F10, a murine melanoma cell line, were obtained from National Centre for Cell Sciences (Pune, India) and were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum. The cultures were sustained at 37° in a humidified incubator containing 5% CO_2. Female C57BL6 mice (6–8-week old) were obtained from Advanced Centre for Treatment, Research, and Education in Cancer (Mumbai, India).

Synthesis of Folic Acid-Modified Cholesterol

The folic acid conjugation to cholesterol was carried out in two steps. In the first step, cholesterol was conjugated to p-aminobenzylamine (PABA), and the cholesterol–PABA conjugate was further reacted with folic acid to obtain the product, CPF. The conjugation of cholesterol to p-aminobenzylamine was carried with some modifications (17). Briefly, cholesterol choroformate (1.13 mmol) and triethylamine (4.44 mmol) were dissolved in chloroform. To this, PABA (122.4 mmol) dissolved in chloroform was added slowly. Addition was carried out at 5–15°C. Reaction mixture was stirred using overhead stirrer (Remi India Ltd., Mumbai, India) for 30 min, at the same temperature; this was followed by 15-min stirring, at room temperature. The reaction was monitored by TLC. Mobile phase used for TLC was hexane:ethyl acetate (95:5). Further, the reaction mixture was washed thrice with water to remove unreacted material, then dried over sodium sulfate (anhydrous), and cholesterol-conjugated p-aminobenzylamine (CP) was isolated by column chromatography using silica gel (200–400 mesh). The mobile phase used for separation by column chromatography was hexane:ethyl acetate (8:2).

Further, the folic acid conjugation of CP was carried out using by a previously reported method with slight modifications (18). Briefly, folic acid (FA) (0.227 mmol) was dissolved in dimethyl sulfoxide (DMSO). To this, dicyclohexyl carbodiimide (DCC) (0.227 mmol) and n-hydroxy succinamide (NHS) (0.227 mmol) were added, and the reaction mixture was stirred for 12 h using a magnetic stirrer, in dark under nitrogen atmosphere, at room temperature.

After 12 h, CP (0.227 mmol dissolved in chloroform and then diluted with DMSO), was added to the above reaction mixture and stirred for another 24 h under the same conditions. Reaction was monitored by TLC. Mobile phase used for TLC was butanol:water:glacial acetic acid (6:1.5:3 ml). Chloroform from the reaction mixture was evaporated on rotary evaporator (Superfit, Mumbai, India). DMSO from the reaction mixture was removed by extracting the product with chilled diethyl ether, followed by evaporation of diethyl ether on rotary evaporator (Superfit, Mumbai, India) to obtain product. The synthesized compounds were confirmed by ^{1}H NMR analysis.

Preparation of Folic Acid-Tagged Liposomal System

Folate-tagged liposomes (FDL) were prepared by remote loading method using ammonium sulfate as described previously by Haran *et al.* (19) with slight modifications. In brief, required quantities of Phospholipon 90G and Phospholipon

90H and cholesterol/folate-modified cholesterol (CPF) (0.9/0.6/0.5) were dissolved in chloroform: methanol mixture (1:1). The lipid solution was evaporated on a rotary evaporator (Buchi India Ltd, Mumbai, India) to remove organic solvents, and thin film was dried under vacuum for 2 h. This formed film was hydrated using ammonium sulfate above the phase transition temperature of the phospholipids (60 ± 2^0) to form the liposomes. Excess ammonium sulfate was removed by centrifugation (21,700 g at $4° \pm 2°C$ for 30 min) (Beckman Coulter, U.S.A.), and desired particle size of liposomes was achieved by probe sonication (Branson India Ltd., Mumbai, India). Liposomes formed were incubated at $60 \pm 2°C$ with DOX solution. Unentrapped DOX was removed by centrifugation (34,000 g at $40 \pm 20°C$ for 45 min) (Beckman Coulter, U.S.A.). The liposomal dispersion was then mixed with 1% poloxamer F127, and the mixture was kept overnight at room temperature. Freeze drying (VirTis bench top model, USA) of the liposomal system was carried out using trehalose as a cryoprotectant. The plain doxorubicin-loaded liposomes (DL), and folate-tagged doxorubicin-loaded liposomes (FDL) were optimized to achieve maximum entrapment and optimum particle size distribution.

Characterization of Delivery Systems

Appearance and DOX Content

The developed systems were characterized with respect to physical appearance, ease of redispersion, and sedimentation behavior. Drug content and DOX leakage after rehydration were estimated by centrifugation at 34,000 g at $4 \pm 2°C$ for 30 min followed by analysis DOX content in the supernatant and pellet by HPLC at 254 nm (Dionex HPLC, India).

Size and Morphology of Liposomes

Particle size was determined by photon correlation spectroscopy on N5 plus submicron particle size analyzer (Beckman Coulter, USA) at 25°C. All measurements were taken by scattering light at 90°C in triplicate.

The liposome morphology was observed using a transmission electron microscope (TEM). Samples for TEM were prepared on formvar-coated 300-mesh size copper grid (SPI suppliers, West Chester, PA, USA). Thin aqueous film blotted with filter paper was prepared with a drop of liposomal suspension. The excess of liposomes was drawn off by tissue paper held at 90° to the plane of grid. The stained grid was air dried and observed. Image was visualized on screen under the electron microscope (Philips CM200, USA) and photographed.

DOX Release from Liposome

The release profile of DOX from liposomes was assessed by the dialysis bag method using USP type I dissolution apparatus in phosphate buffered saline pH 7.4 (50 ml) medium. Reconstituted freeze dried liposomal suspension corresponding to 500 μg of DOX, filled in a dialysis bag (Himedia, India; molecular weight cut off 12,000–14,000 Da) and both ends were tied, was placed in the basket of the dissolution apparatus. RPM and temperature of water bath were set at 50 rpm and $37 \pm 0.5°C$, respectively. The aliquots (0.5 ml) were withdrawn at periodic intervals up to 48 h and analyzed for DOX content by HPLC at 254 nm.

Residual Solvents

The levels of residual organic solvents (methanol and chloroform) within the liposome samples were determined by GC analysis (Varian CP-3800-GC, USA). The analyses were performed using the static head-space method using Varian WSμ software.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) experiments were performed using a differential scanning calorimeter (Mettler Toledo DSC 822, USA). An accurately measured amount of each of the selected freeze dried liposomal samples (5–10 mg) was loaded in an aluminum pan with a central pin hole under dynamic nitrogen (purity: 99.999%, supplier; Mars Gas Ltd, Mumbai, India) purged at 40 mL/min. An empty aluminum pan was used as reference. Pans were sealed using crimper. The sample was heated from 30° to 250°C at a rate of 10°C/min. Measurements were performed in triplicate.

In Vitro Cell Line Studies

Receptor Expression

The expression of the folate receptor on B16F10 cells was evaluated by fluorometry cell uptake assay (20,21). Briefly, monolayer cell culture of B16F10 cells was grown in folate-free DMEM medium. Cells were harvested from subconfluent culture using trypsin EDTA (1%) solution and then suspended in DMEM media containing 10% FBS. An aliquot of 200 μl containing 1×10^5 cells was transferred to two micro centrifuge tubes. In one tube FDL (equivalent to 50 μg/ml of DOX) was mixed with the cells while, in another tube, along with FDL (equivalent to 50 μg/ml of DOX), 100 μl of 1-mM folic acid solution was added. Micro centrifuge tubes were then incubated at 37°C and 5% CO_2 for 2 h with intermittent shaking after every half hour. After treatment, the cells were washed three times with phosphate buffered saline (PBS) and lysed with 1% Triton X-100 solution, and the fluorescence intensity of DOX associated with the cells in each tube was measured on Synergy^{HT} multidetection microplate reader (BioTek Instruments Inc. Winooski, USA). The excitation and emission wavelengths were set at 530 and 590 nm, respectively. Cellular uptake of the liposomal DOX formulations was quantified by the fluorescence intensity.

Cell Uptake Process

To evaluate the targeting potential of developed delivery systems *in vitro*, cell uptake studies of DOX, poloxamer-coated untagged DL, and FDL were carried out in B16F10 melanoma cells at a final DOX concentration of 100 μg/ml. For uptake of the delivery systems and DOX, the process followed was same as described for receptor expression (Appearance and DOX Content section).

Cell uptake study was also qualitatively performed. Briefly, monolayer cell culture of B16F10 cells was grown in folate-free DMEM medium. Cells were harvested from subconfluent culture using trypsin EDTA (1%) solution and suspended in folate-free DMEM medium containing 10% FBS. Cell suspension was diluted appropriately according to cell count. An aliquot of 500 µl containing 2×10^5 cells was transferred on autoclaved cover slip placed in each well of six-well tissue culture plate. After this, 3.5 ml of DMEM medium was added, to each well of the culture plate, and incubated overnight at 37°C and 5% CO2. After incubation, the medium was replaced from each well with 200-µl medium containing various delivery systems along with FDL, DL, and DOX, and was mixed with the cells at a final DOX concentration of 50 µg. The culture plate was then incubated at 37°C and 5% CO2 for 2 h. After incubation, medium was removed, and cells were washed three times with PBS. Cover slips containing treated cells were taken out from the culture plate and observed under the inverted microscope with fluorescence attachment (Zeiss, Germany).

Optimization of Ligand Content in Delivery System

To evaluate the optimum concentration of folate in the targeted delivery system *in vitro*, cell uptake studies of the tagged delivery system were carried out with varying concentrations of ligand in the delivery system. Liposomes with varying concentrations of folate were prepared (0.5, 1, and 2%) and subjected to cell uptake studies as describe in Appearance and DOX Content section.

Cytotoxicity Assay

The cytotoxicity assay was carried out in A549 human lung cancer cell lines and B16F10 murine lung carcinoma cell lines (22).

A549 cells and B16F10 cells (1.0×104 cells/well) were seeded in a 96-well plate and grown in DMEM containing 10% FBS (v/v) overnight at 37°C and 5% CO_2. Free DOX and DOX-liposome (DL and FDL) were added, and the cells were incubated at 37°C and 5% CO_2 for 48 h. After incubation, cell viability was evaluated by assay with MTT [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salts]. Medium from each well was removed, and MTT (0.5 mg/ml) was added to each well. Plate was incubated for another 4 h at 37°C and 5% CO_2. MTT solution from each well was then replaced by 10% sodium dodecyl sulfate solution (SDS) to dissolve the formazan crystals converted from MTT by live cells. The plate was then read on Synergy^{HT} multidetection microplate reader (BioTek Instruments Inc. Winooski, USA), and cell viability was determined from absorbance at 570 nm. Plots of percent cell growth inhibition *vs*. concentration of DOX were plotted, and IC_{50} values were calculated.

In Vivo Studies

Acute Toxicity Studies

The study was approved by independent Animal Ethics Committee of Bombay College of Pharmacy, and experiment was performed as per the OECD guidelines. Swiss Albino mice (25–35 g) were administered with DOX and FDL by IV route (dose, 6 mg/kg) through tail vein. Animals were observed and monitored over a period of 2 weeks for mortality, body weight, and any abnormal behavior such as tremors, convulsions, salivation, and diarrhea. They were sacrificed on the 14th day; weight of all vital organs was taken, and histopathological evaluation of heart tissue was carried out.

Biodistribution Studies

Radiolabeling of the Delivery System. Untagged and tagged liposomes were labeled with ^{99m}Tc after reduction with stannous chloride ($SnCl_2$). Briefly, freeze dried liposomes (DL and FDL) were dispersed in saline, and 0.1 ml of $SnCl_2$ in 0.01 M HCl was added to it. Mixture was shaken well, followed by addition of 0.1 ml of ^{99m}Tc (~3 mCi) in saline. The reaction mixture was incubated for 10 min at room temperature. Labeling efficiency was evaluated by thin layer chromatography (TLC). The radiolabeled formulation was spotted on a TLC plate and run using saline as the mobile phase. The activity was measured using well-type gamma counter (Electronic Corporation of India, Mumbai). The percent radiolabeling efficiency was represented as percent ratio of bound activity to total activity. Stability of radiolabeled complex in mice plasma was determined by monitoring the radioactivity up to 24 h at ambient temperature.

Animal experimentations (biodistribution and efficacy study) were approved by Bhabha Atomic Research Centre Animal Ethics Committee, RMC, Parel, Mumbai, India. Study was carried out by protocol similar to that described by Peer and Margalit 2004 with slight modification (23). Briefly, female C57BL6 mice were randomly divided into three groups ($n = 5$). B16F10 murine lung carcinoma cells (1×10^6 cells in 100 µl of PBS) were injected by subcutaneous route to the right armpit of mice, except one group (non-tumor-bearing). Tumors were allowed to develop up to the palpable size in two groups (tumor-bearing). The tumor-bearing groups were then injected with radiolabeled formulations (DL and FDL—a dose of 6 mg/kg body weight) through tail vein., where as non-tumor-bearing group received radiolabeled FDL formulation. After 24 h, all animals were sacrificed by using over dose of thiopentone sodium anesthesia (200 mg/kg of body weight by i. p. route). Necropsy was performed, and all vital organs along with injection site of solid tumors were collected. Radioactivity pertaining to each organ was measured by using gamma scintillation counter (Electronics Corporation of India, Mumbai, India). The percent uptake was calculated as ratio of radioactivity count of the organ to the total radioactivity administered.

Efficacy Studies

The study protocol was adopted from the report by Hwang and Peer (23,24) with slight modification. Tumor was developed in C57BL6 mice by injecting B16F10 murine lung carcinoma cells (1×10^6 cells in 100 µl of PBS) subcutaneously to the right armpit of each animal. Mice were then separated into different groups; formulations (DL and FDL), DOX,

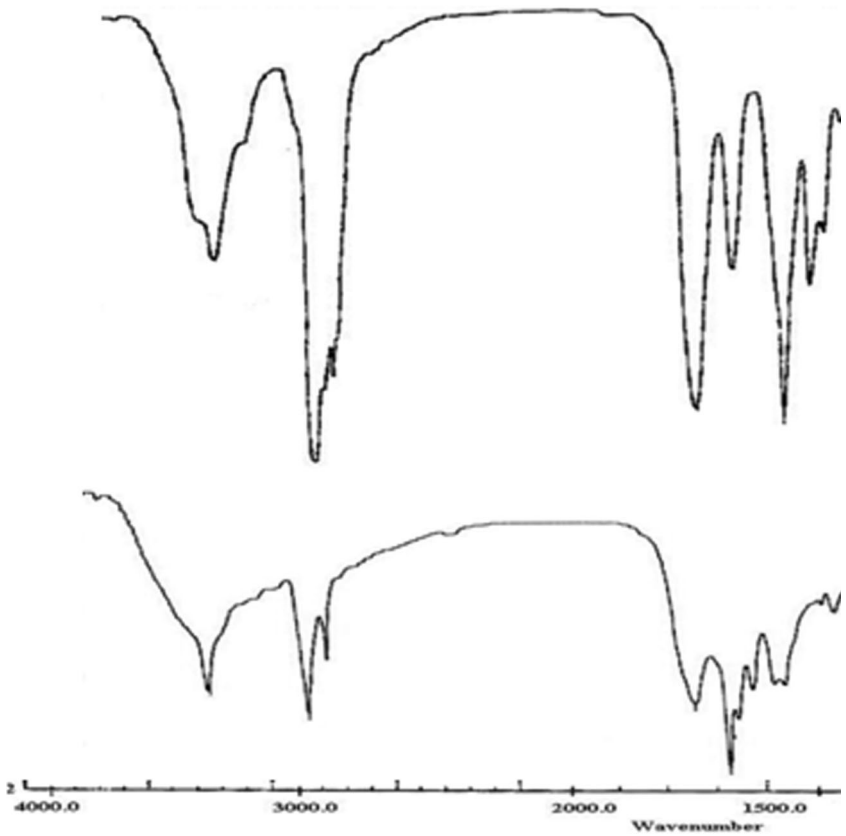

Fig. 1. FTIR spectra of CP and CPF

control, and blank liposomes, and respective formulations were injected (dose 6 mg/kg) through tail vein by i.v. route. Animals were observed and monitored for mortality and any abnormal behavior such as tremors, convulsions, salivation, and diarrhea until the completion of the study. Twelve days after dosing, mice were sacrificed. Tumors were separated and weighed. Tumor regression was calculated by the weight of the tumor, the higher the weight of the tumor, the lesser the regression. Efficacy of the delivery system was represented by regression in tumor weight and effect on RBC count.

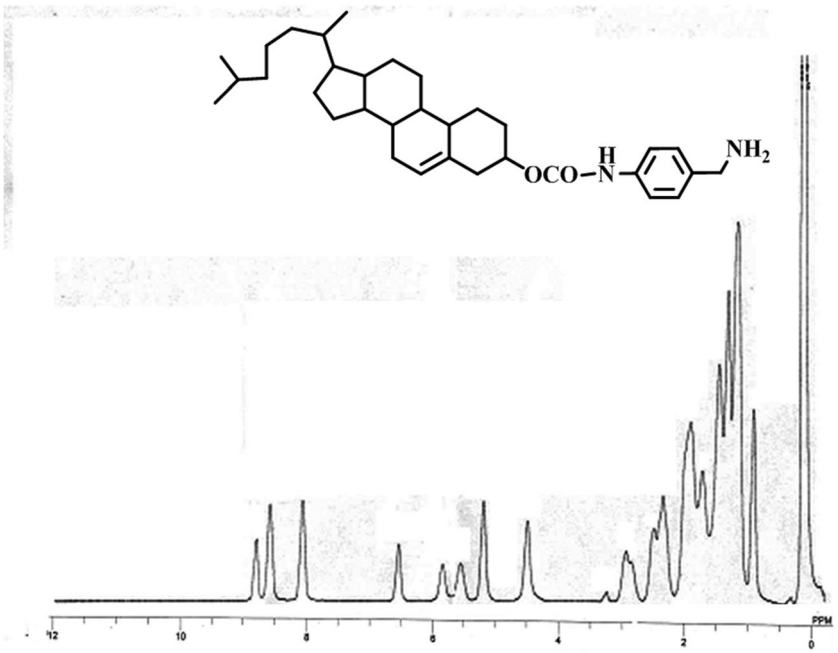

Fig. 2. NMR spectra of CP

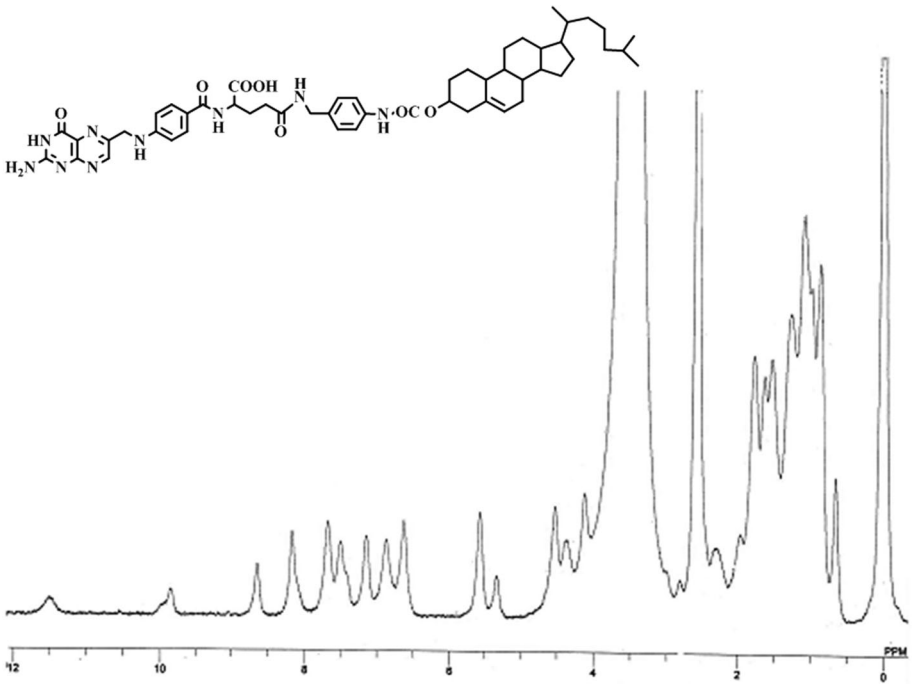

Fig. 3. NMR spectra of CPF

Statistical Analysis of Data

All data are reported as mean ± standard deviation (SD), and differences between the groups were tested using Student's *t* test at the level of significance of p value <0.05.

RESULTS

Synthesis and Characterization of Folic Acid-Conjugated Cholesterol

The reaction was covalent conjugation of cholesterol chloroformate with p-aminobenzylamine (CP) followed by conjugation of CP complex with folic acid (CPF) (17,18). FTIR was used to confirm the formation of the products. FTIR spectra of synthesized CP and CPF are depicted in Fig. 1a, b. In Fig. 1a, the peak at 3358 cm^{-1} corresponds to NH group, where as those at 1626 and 1518 cm^{-1} correspond to amide group, and peak at 1699 indicates urethane group. Figure 1b depicts the FTIR spectrum of the CPF in which 1575 and 1535 cm^{-1} can be attributed to amide group, and 1626 cm^{-1} corresponds to secondary NH group. The structures of CP and CPF were further confirmed by 1H NMR, as shown in Figs. 2 and 3, respectively. The NMR spectra show characteristic signals which confirmed the formation of the compounds, *viz* CP and CPF.

Table I. Chemical Composition of Uncoated Liposomes

Composition	Untagged liposomes (DL) (Mol%)	Folic acid tagged liposomes (FDL) (Mol%)
Phospholipon 90G	52.3	52.3
Phospholipon 90H	34.8	34.8
Cholesterol	12.9	11.9
CPF	–	1.0

Preparation and Characterization of Delivery Systems for DOX

The liposomal formulation of DOX was prepared by active loading using ammonium sulfate gradient method. FDL were prepared by insertion of folate-conjugated cholesterol (CPF) in the preparation of liposomes. The entrapment of DOX was 3028 μg/135 mg of lipid. Table I reports the composition of liposomal formulations. The physicochemical properties of the liposomal formulations namely DOX content, size, and residual solvent levels are given in Table II. The residual levels of chloroform and methanol in the developed liposomal formulations were found to be very low, well below the ICH limits.

Table II. Physicochemical Characterization of FDL

Characteristic	Folic acid tagged liposomes (FDL)
Drug content (μg/135-mg lipid)	3028 ± 59.6
Visual appearance and ease of redispersion	Red-colored powder readily dispersed in saline
Particle size (nm) before freeze drying	194.6 ± 14.6
Particle size (nm) after freeze drying	300.9 ± 16.9
Drug leakage (%)	3.76 ± 0.08
Organic solvent residue (ppm)	Methanol—1 ppm, chloroform—not detected

All data is with ±SD; $n = 3$

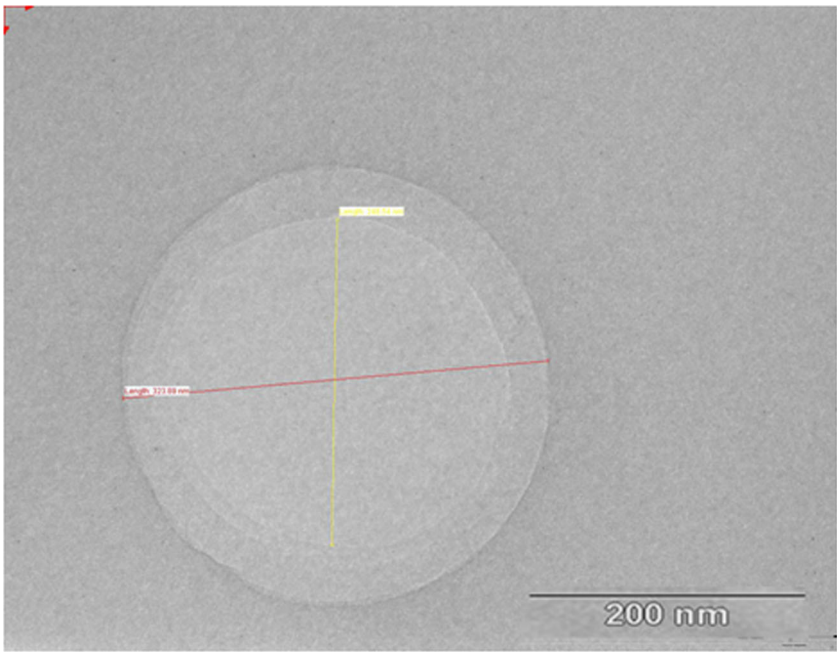

Fig. 4. Transmission electron microscopy image of FDL

TEM was used to image the DOX liposomes and to assess their size, shape and lamellarity. The DOX-loaded liposomes appear as bright spheres surrounded by dark thick layer showing large internal aqueous core surrounded by one or two lamellae (Fig. 4). Mean particle sizes obtained from TEM photomicrographs were in the range of 200–400 nm, and size obtained by TEM was close to the size measured by photon correlation spectroscopy (Table II).

DSC scan of DOX (DSC thermogram A in Fig. 5) indicated the onset of endotherm at 216.61°C and peak at 234.17°C. Blank liposomes (DSC thermogram D in Fig. 5) showed the endotherm peak at 94.36°C, whereas in DL, FDL (DSC thermograms B and C, respectively in Fig. 5) showed the onset of endotherm at 206.09°C and attainment of peak at 210.85°C. Thus, the DSC study of the developed liposomal formulations revealed the presence of the DOX in

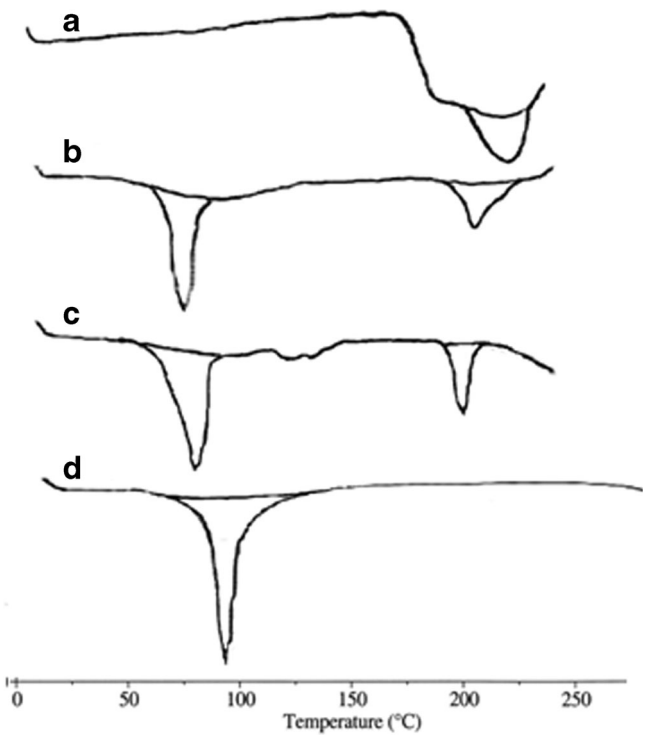

Fig. 5. DSC thermograms (**a** DOX, **b** DL, **c** FDL, and **d** blank liposomes)

the liposomal system. *In vitro* DOX release from liposomes (Fig. 6) suggested that there is burst release of DOX (up to 28%) for initial 10 h after which there is very small increase in release over the period of 48 h (up to 30%).

In Vitro Cell Line Evaluation

Receptor Expression

Folic acid competition assay was carried out to establish the presence of folic acid receptor on the surface of B16F10 cell line. The uptake of DOX from folate-targeted delivery system was reduced from 25.42 ± 0.89 μg DOX/million cells to 19.19 ± 1.07 μg DOX/million cells in presence of 1 mM folic acid (Fig. 7). The assay thus revealed the inhibition of uptake of the FDL system into B16F10 cells due to the presence of excess folic acid, indicating the presence of folic acid receptors on the surface of the B16F10 cells, which is responsible for the uptake.

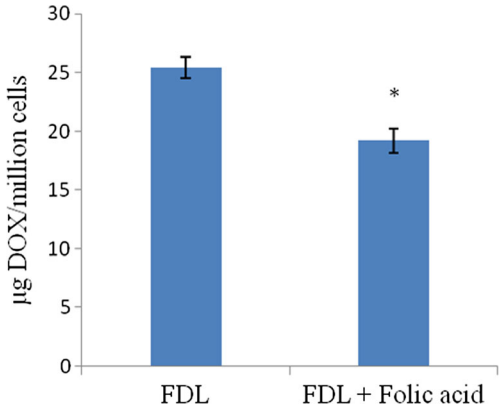

Fig. 7. Folic acid receptor expression assay in B16F10 cell. Uptake of folate-tagged DOX liposomes (equivalent to 50 μg/ml of DOX) was evaluated by fluorometry with or without presence of free folic acid (1 mM folic acid). Uptake of folate-tagged DOX liposome (FDL) was significantly reduced in presence of free folic acid. *Students *t* test *p* value <0.05

Cell Uptake Process

Cellular uptake study indicated an increased uptake of FDL (25.42 ± 0.89 μg DOX/million cells) as compared to that of DL (17.99 ± 1.30 μg DOX/million cells) and DOX (10.58 ± 1.21 μg DOX/million cells) (Fig. 8) due to presence of folate receptor on the cell surface, which promoted the uptake of FDL.

The results of fluorescence microscopy also suggested that there is an increase in accumulation of DOX inside the cells, in the case of FDL as compared to the conventional DL and DOX. The trend in accumulation of drug is as follows: FDL > DL > DOX.

Optimization of Ligand Content in Delivery System

Optimization of percent ligand content was carried out by measuring the uptake of the liposomal delivery system (FDL) with varying folate content in B16F10 cells. Results revealed increased uptake of DOX from 0.5 Mol% (21.89 ± 2.32 μg) to 1.0 Mol% (25.42 ± 1.98 μg) folate containing targeted delivery system due to presence of excess folate on the delivery system (Fig. 9). However, from 1.0 to 1.5 Mol% folate-targeted delivery system, there is not much increase in the folate uptake (25.9 ± 1.54 μg). This may be attributed to the saturation of the folate receptors.

Cytotoxicity Assay

MTT-based cytotoxicity assay was performed to compare the anticancer effect of the folate-targeted delivery systems against the A549 human lung cancer cells and B16F10 murine lung cancer cells. The IC_{50} values for DOX, DL, and FDL were very close 1.36–2.10 μM for A549 and 0.927–1.315 μM for B16F10 cell line. This may be attributed to the fact that DOX is slowly released from the liposomal formulation. Also, the incubation time was 48 h, which is considered to be small, and possibly, there is incomplete release of DOX from the delivery system. However, folate-conjugated delivery system had shown a marginally improved inhibitory potential as compared to untagged delivery system as shown in Table III.

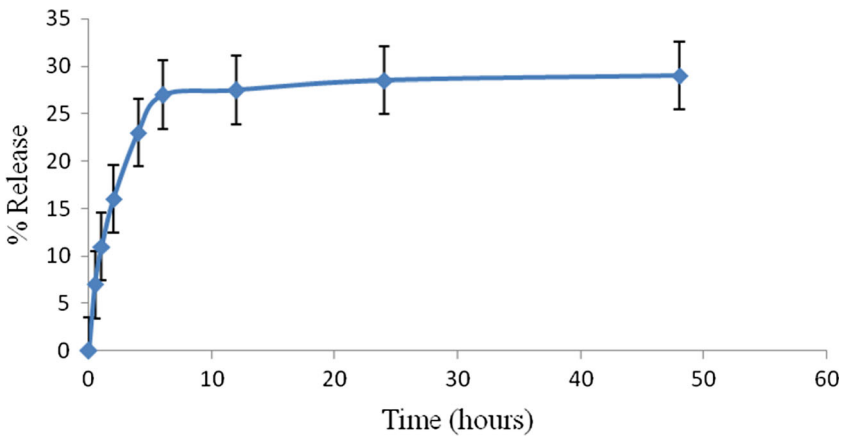

Fig. 6. *In vitro* drug release from FDL (reconstituted liposome filled in dialysis bag) in phosphate buffered saline pH 7.4 medium using USP type I dissolution apparatus at 50 rpm and 37 ± 0.5°C

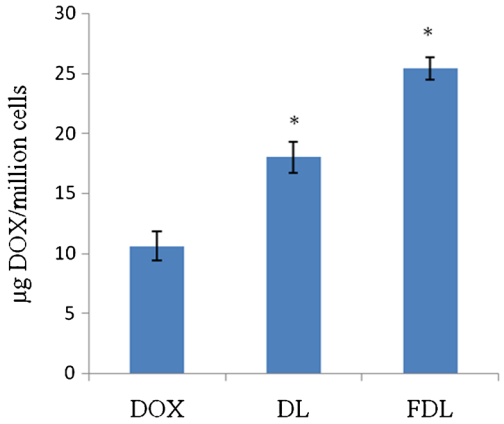

Fig. 8. Cell uptake study in B16F10 cell line. Uptake of folate-tagged DOX liposomes (equivalent to 100 µg/ml of DOX) was evaluated by fluorometry in folic acid-free media. Uptake of folate-tagged DOX liposome (FDL) was significantly higher as compared to untagged DOX liposome (DL) and free DOX. *Students *t* test *p* value <0.05

In Vivo Studies

Acute Toxicity Studies

Swiss Albino mice were administered with DOX and folate-tagged liposomes by IV route (dose, 6 mg/kg) through tail vein. No changes in the skin, fur, eyes, and behavioral pattern were observed in any of the dosed animals. The animals of all the groups survived for the complete duration of the study. There was no significant difference in the increase in body weights and mean relative organ weights (Table IV) of the animals of the control group and the treated groups. However, histopathology studies of the heart revealed severe cardiotoxicity of animals treated with free DOX as compared to animals treated with FDL and the control group. Free DOX-treated group showed cytoplasmic vacuolation and loss of muscle striations with severe pathology, whereas FDL-treated group showed only mild pathological changes as compared to the control group.

Table III. MTT Assay of Delivery Systems

Delivery system	IC50 A549 (µM)	IC50 B16F10 (µM)
Doxorubicin	1.36	0.927
DL	2.23	1.404
FDL	2.10	1.315

Biodistribution Study

Radiolabeling of the Liposomes. Freeze dried DL and FDL liposomes were reconstituted in saline and were radiolabeled with Technetium-99 m (^{99m}Tc), using stannous chloride as reducing agent, Radiolabeling conditions were optimized with respect to time of incubation and ratio of ^{99m}Tc: liposomes. Radiolabeling efficiency of the liposomal system was found to be more than 98%. The optimum ratio of ^{99m}Tc: liposomes was found to be 3 mCi:25 mg, while optimum incubation time was found to be 10 min. The developed radiolabeled complex was stable for 24 h in saline at 37 ± 0.5°C.

Biodistribution study was performed by injecting radiolabeled liposomal formulation through tail vein in tumor-bearing C57BL6 mice; gamma scintigraphic biodistribution profiles of DL and FDL are depicted in Fig. 10. FDL has shown different pattern of uptake compared to that of DL delivery system. FDL has shown reduced uptake of liposomes in the liver and spleen, and increased uptake to significant level in tumor (*p* value <0.05).

In Vivo Efficacy Study in Solid Tumor Model

Tumor-bearing C57BL6 mice were sacrificed 12 days after a single dose of either DOX or formulations, and tumor weight was taken as measure of regression in tumor growth. The tumor volume was reduced in the treatment group as compared to the control untreated group (Figs. 11 and 12). The growth of tumor in mice treated with FDL was

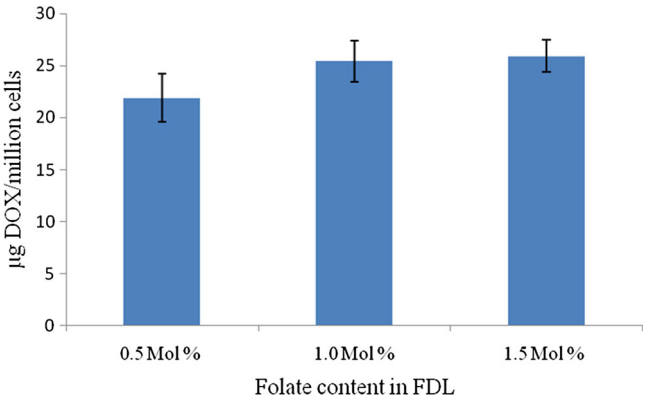

Fig. 9. Optimization of folate content by (liposomes prepared with varying content of folic acid 0.5, 1.0, and 1.5 Mol% containing equivalent to 100 µg/ml of DOX) in delivery system by cell uptake study in B16F10 cell line

Table IV. In Vivo Acute Toxicity Results in Swiss Albino Mice

Group	% gain in body weight	% mean relative organ weight				
		Lung	Heart	Kidney	Spleen	Liver
Control	9.0 ± 0.15	0.95 ± 0.05	0.42 ± 0.05	1.03 ± 0.07	0.49 ± 0.06	5.21 ± 0.06
Blank	10.1 ± 0.19	1.02 ± 0.06	0.43 ± 0.04	1.10 ± 0.06	0.44 ± 0.02	5.90 ± 0.08
DOX	7.8 ± 0.22	0.91 ± 0.07	0.42 ± 0.08	1.08 ± 0.09	0.45 ± 0.06	5.32 ± 0.09
FDL	10.1 ± 0.21	0.92 ± 0.06	0.43 ± 0.04	1.05 ± 0.08	0.55 ± 0.05	5.45 ± 0.06

All data is with ±SD; $n = 3$

significantly inhibited when compared to those treated with DL and free DOX (p value <0.5). The effect of developed FDL systems on RBC count was studied, and results revealed (Fig. 13) less reduction of RBC count in FDL-treated mice as compared to the DL-treated and DOX-treated group.

DISCUSSION

Liposomal systems are increasingly being used for delivery of anticancer agents because of their ability to accumulate in the tumor cells due to the enhanced permeation and retention (EPR) effect of cancer cells. In addition, the surface of liposomes can be modified with ligands for receptors over expressed on cancer cells; hence, active targeting is also possible. Previous studies demonstrated that the folate receptor is highly expressed in cancer cells (5–10). Therefore, this membrane receptor appears as an attractive target to develop appropriate folate-based drug delivery systems for anticancer therapy. Several attempts in the past have been tried for active targeting using folic acid as targeting ligand by conjugation of folic acid to lipid or polymer using PEG as linker (13–15). The present study aimed to explore the use of p-aminobenzylamine (PABA) as a linker which provides versatility of anchoring targeting ligands to delivery system. Conjugation of folic acid with cholesterol was carried out using PABA and then subsequent incorporation of folate-tagged cholesterol in the liposomes. Cholesterol chloroformate was linked to p-aminobenzylamine, and this complex was then further reacted with folic acid by amidation reaction to give cholesterol-linked folic acid-targeted conjugate (Fig. 14). These synthesized compounds were then incorporated into liposomal system to evaluate the targeting potential of folic acid tagged systems.

In the present work, DOX was used as a model drug for the evaluation of folate-based bioconjugated liposomal delivery system against solid tumors. DOX is a widely used anticancer agent and is active against various types of cancers. DOX is reported with severe toxicity like cardiomyopathy, secondary malignancies, extravasation and tissue necrosis, and severe myelosuppression (25). There are two liposomal formulations of DOX, which are approved for clinical use; these are Myocet® and Doxil®. DOX loading in liposomes is generally carried out by remote loading method, which is based on the concentration gradient.

In the present investigation, ammonium sulfate gradient method was used for DOX loading. The formulation and process of preparation of liposomes were optimized with respect to composition of phospholipids and cholesterol, sonication time, incubation time, and cryoprotectant ratio so as to achieve maximum DOX entrapment and optimum particle size.

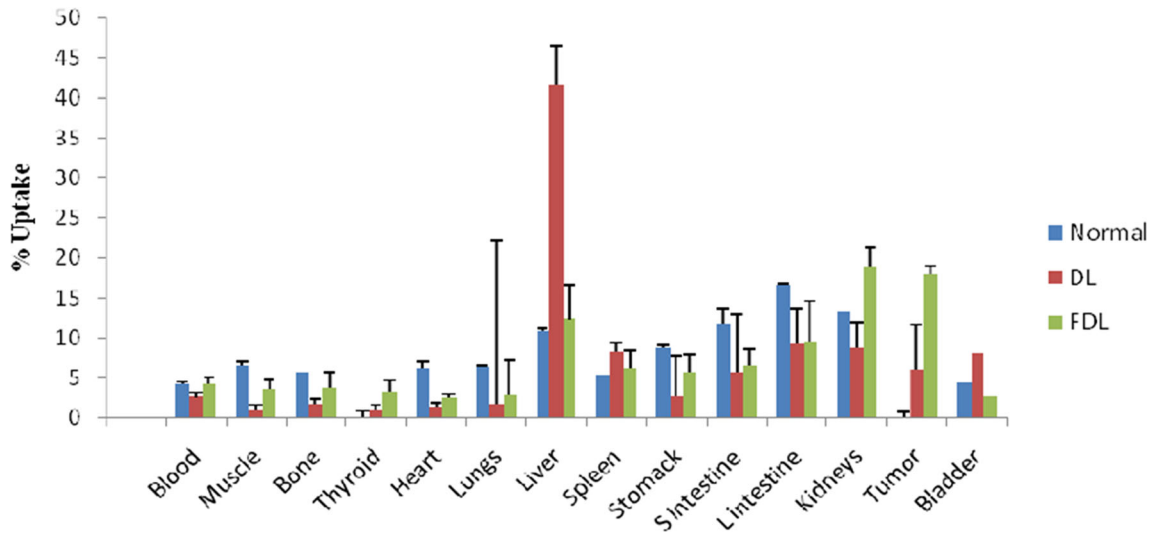

Fig. 10. *In vivo* biodistribution study. Percent radioactivity in various organs of solid tumor-bearing C57BL6 mice 24 h after IV administration of ^{99m}Tc-tagged folate-conjugated liposome (FDL) and untagged (DL) delivery system (dose, equivalent to 6 mg/kg of DOX) and percent radioactivity in various organs of non-tumor-bearing C57BL6 mice (normal) 24 h after IV administration of ^{99m}Tc-tagged DOX (dose, 6 mg/kg)

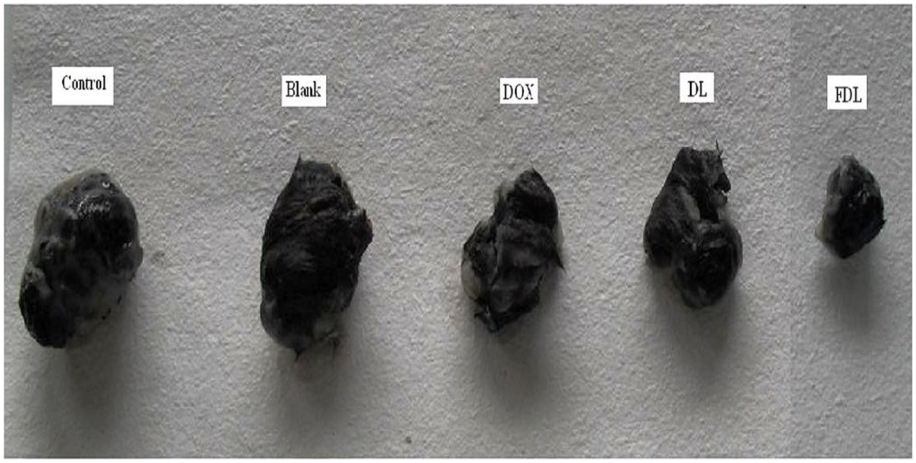

Fig. 11. Pictographical representation of tumor efficacy study carried out in solid tumor-bearing C57BL6 mice. Representative tumors 12 days after a single dose of blank liposome, FDL, DL, and DOX (6 mg/kg)

It is well known that covering colloidal particles with hydrophilic, nonionic polymers such as polyethylene glycol (PEG) or poloxamer greatly increase their circulation time—by steric stabilization of the colloidal delivery system—and avoid the reticuloendothelial system (RES) uptake. Surface stabilization of liposomes can be achieved by adsorption of coating polymer on the surface of the colloidal delivery system or by the covalent-linked polymer incorporated into the delivery system (13,26–28). In the present study, liposomes were coated with 1% poloxamer F127 by physical adsorption method prior to freeze drying. The particle size of DL liposomes was increased from 194 to 300 nm, indicating the coating of liposomes.

To improve the stability of liposomes, they were freeze dried. However, freeze drying process can lead to destruction of membrane function of the liposomes, substantial leakage of the entrapped compound, which can be controlled and reduced by using appropriate cryoprotectant while freeze drying. Trehalose was used as cryoprotectant as it had shown less DOX leakage (less than 4%), and vesicle size was also not affected to a great extent. Different ratios of trehalose:lipid were tried, and 4:1 was found to be most effective in terms of maintaining particle size as well as avoiding DOX leakage.

The freeze dried formulation appeared as red-colored fluffy powder, which was easily redispersed upon reconstitution in sterile saline. *In vitro* release study of the liposomes was carried out to understand percent DOX leakage from the delivery system in PBS pH 7.4; results suggested that there is a burst release of DOX (up to 28%) for initial 10 h after which there is very small increase in release over the period of 48 h (up to 30%). The developed delivery system is required to release DOX after reaching target site, *i.e.*, tumor. From *in vitro* release pattern, it can be inferred that only 30% of DOX is released in plasma and around 70% of DOX reaches the target tumor cells.

In vitro cell line studies were carried out in A549 human lung cancer cell lines and B16F10 murine lung carcinoma cell lines. The receptor expression assay carried out in B16F10 cell line confirmed the presence of folic acid on cell surface, as the uptake of folate-conjugated liposomes was reduced. This

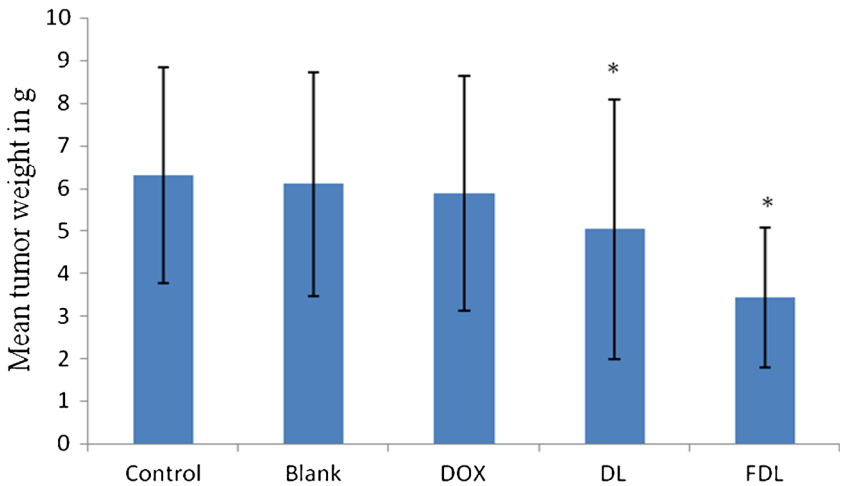

Fig. 12. Mean tumor weight analysis ($n = 5$) in solid tumor-bearing C57BL6 mice. Tumor weights 12 days after a single dose of blank liposome, FDL, DL, and DOX (6 mg/kg). *p value <0.05 (t test)

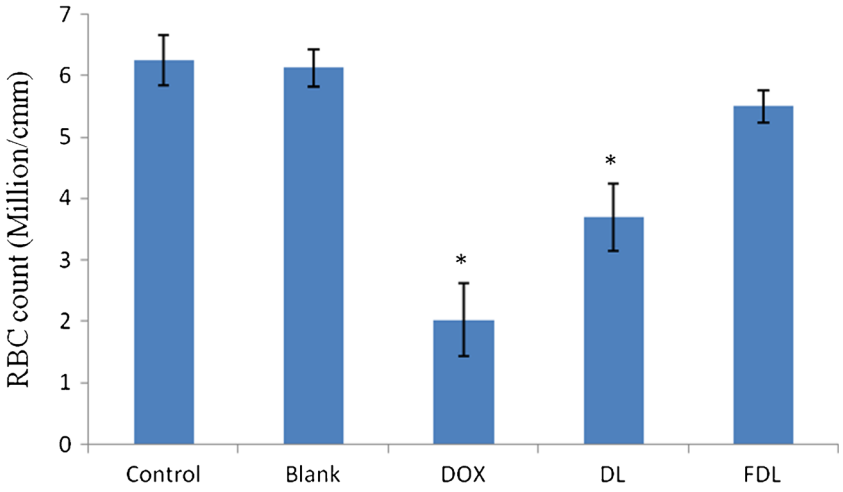

Fig. 13. Effect on RBC in solid tumor-bearing C57BL6 mice. RBC count 12 days after a single dose of blank liposome, FDL, DL, and DOX (6 mg/kg). *p value <0.05 (t test)

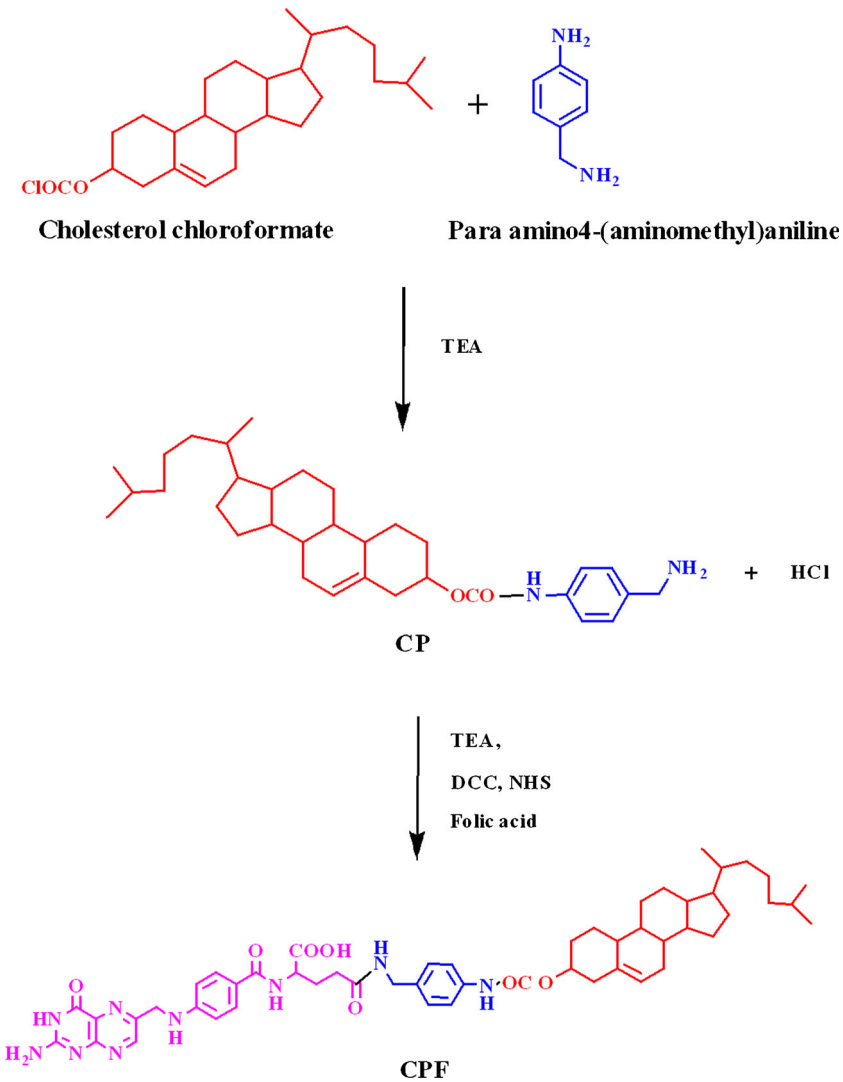

Fig. 14. Schematic representation of preparation of cholesterol-conjugated folic acid. Conjugation of cholesterol chloroformate to p-aminobenzylamine and then further reaction with folic acid

indicates the competitive inhibition of receptor-binding site on the surface by folic acid and resulting in reduced uptake of folate-conjugated delivery system in the cell. Similarly, in cell uptake studies, the uptake of FDL was increased as compared to conventional DL and free DOX (20).

In vitro cytotoxicity studies carried out in A549 human lung cancer cell lines and B16F10 murine lung carcinoma cell lines revealed enhanced uptake of FDL as compared to DL. Acute toxicity study in healthy mice did not show any signs of toxicity after administration of single dose of formulation as compared to DOX which is reported to be highly cardiotoxic. Thus, as reported previously, (29) our studies have also shown that the liposomal formulation is able to reduce toxicity. Biodistribution study in solid tumor-bearing C57BL6 mice showed that FDL is taken up to a greater extent in tumor as compared to DL. Also, FDL showed reduced uptake in the liver and spleen, and uptake increased to a significant level in tumor. Similar observations were recorded by Low *et.al*, in an uptake study of PEG-folate-tagged liposomal system, wherein uptake in tumor was increased due to folate tagging to the liposomal delivery system (30). The tumor growth in subcutaneously administered B16F10 melanoma-bearing mice treated with FDL was inhibited to a greater extent, when compared with those treated with conventional DL liposomes and free DOX. Therefore, these results indicate that FDL exhibits inhibition of tumor growth more efficiently than conventional DL liposomes. Myelosupression is one of the major side effects of DOX (31), which is reduced markedly in the FDL treated groups, evidenced by the RBC counts. The results showed that the targeted FDL liposomes showed significantly better protection with less toxicity as compared to conventional DL liposome and free DOX.

Through these experiments, it is demonstrated that FDL is selectively internalized into FR-expressing tumor cells including B16F10 and A549 cells as well as B16F10 melanoma-bearing mice. Moreover, we have highlighted that FDL will lead to a reduction in toxicity of DOX. Although, we have shown the importance of the folate targeting, further experiments with more cell lines and chronic toxicity and efficacy studies in higher animals should be conducted to fully realize the potential of these systems.

CONCLUSION

The present investigation had suggested that application of the p-aminobenzylamine as linker for conjugation of ligands like folic acid on cholesterol. Such conjugation provides versatility and enables the researchers to anchor different targeting ligands with simple reaction procedures. Further, such conjugated lipid can be easily incorporated in the liposomal drug delivery systems. The study has conjugated folic acid as a ligand, and liposomal systems prepared using it has shown good targeting potential to cancer cells. Further investigations in various types of tumors-expressing folic acid will ensure the merit of the developed folate-targeted liposomal system.

ACKNOWLEDGMENTS

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COMPLIANCE WITH ETHICAL STANDARDS

All the animal studies were approved by independent ethics committee of Bhabha Atomic Research Centre, Mumbai, India. All the procedures involved in the animal studies were performed in accordance with the recommendations of NIH guidelines for the proper use and care of laboratory animals. In addition, the study was approved by independent Animal Ethics Committee of Bombay College of Pharmacy, and experiment was performed as per the OECD guidelines.

Conflict of Interest The authors declare that they have no competing interests.

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