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Research Article

Nanocarrier-Based Combination Chemotherapy for Resistant Tumor: Development, Characterization, and *Ex Vivo* Cytotoxicity Assessment

Sarjana Raikwar,[1] Sonal Vyas,[2] Rajeev Sharma,[1] Nishi Mody,[1] Surbhi Dubey,[1] and Suresh P. Vyas[1,3]

Abstract. A folic acid-conjugated paclitaxel (PTX)-doxorubicin (DOX)-loaded nanostructured lipid carrier(s) (FA-PTX-DOX NLCs) were prepared by using emulsion-evaporation method and extensively characterized for particle size, polydispersity index, zeta potential, and % entrapment efficiency which were found to be 196 ± 2.5 nm, 0.214 ± 0.04, $+23.4 \pm 0.3$ mV and $88.3 \pm 0.2\%$ (PTX), and $89.6 \pm 0.5\%$ (DOX) respectively. *In vitro* drug release study of optimized formulation was carried out using dialysis tube method. FA-conjugated PTX-DOX-loaded NLCs showed 75.6 and 78.4% (cumulative drug release) of PTX and DOX respectively in 72 h in PBS (pH 7.4)/methanol (7:3), while in the case of FA-conjugated PTX-DOX-loaded NLCs, cumulative drug release recorded was 80.4 and 82.8% of PTX and DOX respectively in 72 h in PBS (pH 4.0)/methanol (7:3). Further, the formulation(s) were evaluated for *ex vivo* cytotoxicity study. The cytotoxicity assay in doxorubicin-resistant human breast cancer MCF-7/ADR cell lines revealed lowest GI_{50} value of FA-D-P NLCs which was 1.04 ± 0.012 μg/ml, followed by D-P NLCs and D-P solution with GI_{50} values of 3.12 ± 0.023 and 3.89 ± 0.007 μg/ml, respectively. Findings indicated that the folic acid-conjugated PTX and DOX co-loaded NLCs exhibited lower GI_{50} values as compared to unconjugated PTX and DOX co-loaded NLCs; thus, they have relatively potential anticancer efficacy against resistant tumor.

KEYWORDS: Nanostructured lipid carrier(s); Paclitaxel; Doxorubicin; Folic acid; resistant tumor.

INTRODUCTION

Cancer is a leading cause of deaths and a serious health problem in both the developing and developed world [1]. Conventional therapies, such as, radiotherapy, chemotherapy, and the combination of both radiotherapy and chemotherapy, are considered to be the primary treatments in cancer patients [2]. However, the systematic administration of non-specific anticancer drugs causes significant toxicities and undesirable side effects due to anticancer agents used which affect the normal cells as well [3, 4]. The main curative therapies for cancer such as surgery, radiation, and chemotherapy are generally successful if the cancer is detected at an early-metastatic stage; otherwise, these treatments remain to be of limited use. When multidrug resistance developed in a cancer cell, it leads to failure of single anticancer drug-based therapy. The limitation of chemotherapeutic agents is multidrug-resistant (MDR) phenotypes, which is a major obstacle to cancer treatment. The MDR is associated with acquired defense mechanisms, for instance blocked apoptosis, increased drug efflux, and decreased uptake of drug [5, 6]. The use of combination chemotherapy for resistant cancer is a more promising approach. Combination chemotherapy is one of the strategies used to overcome the cancer multidrug resistance, where in the drugs have their different molecular targets for pharmacodynamic activity and hence result in an improved therapeutic outcome either by increasing therapeutic efficacy or reducing toxicity [7–10]. It has been reported that the co-administration of anticancer drugs to tumor cells using nanocarriers is an optimistic strategy for treating MDR [11, 12, 6]. The combination therapy of two or more drugs promotes synergism among different drugs against cancer cells and averts drug resistance owing to different but distinct mechanism of action of drugs taken in combination. Paclitaxel and doxorubicin combination is considered as a favorable and effective regimen in the first-line treatment of many solid tumors, where paclitaxel promotes the assembly of microtubules

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Abbreviations P, paclitaxel; D, doxorubicin; FA, folic acid; FA-D-P NLCs, folic acid-doxorubicin-paclitaxel nanostructured lipid carrier(s) formulation; D-P NLCs, doxorubicin-paclitaxel nanostructured lipid carrier(s) formulation; B-NLCs, plain nanostructured lipid carrier(s) formulation; D-P- Sol, doxorubicin-paclitaxel solution; D-sol, doxorubicin-solution; P-sol, paclitaxel solution; ADR, adriamycin

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and reversibly binds to β-tubulin resulting into polymerization of microtubules while doxorubicin exhibits its cytotoxic effect through intercalation and formation of an inclusion complex with DNA strands and also an inhibitory complex with topoisomerase II. If two desirable drugs are combined in NLCs, they could be co-delivered to the tumors for controlled release with an optimized pharmacokinetic profile. NLCs are defined as alternative lipidic nanoparticles which consist of blend of spatially different lipids, that is, solid lipid(s) with liquid lipid(s), where the amount of liquid lipid can be varied to attain controlled release of the drug. This blend forms a typical crystalline structure with many imperfections which provide more space for drug loading [13]. NLC-based drug delivery is advantageous particularly at systemic level, and the apparent therapeutic advantages include longer circulation half-life, improved pharmacokinetic, and reduced side effect. The combination chemotherapy is capable of delivering large amount of drug to achieve and maintain therapeutic concentration over a longer period of time at a target site. Sharma *et al.* have provided a comprehensive review on drug targeting to tumor site and available modalities for various applications in theranostic in cancer therapeutics [14]. The targeted drug delivery system is used for the selective delivery of a pharmacologically active drug at pre-identified site in a therapeutic concentration, with restricted access to non-target sites, thereby minimizing the toxic effects with an optimum therapeutic outcome [15]. Raniolo *et al.* reported the selective targeting of nanocarriers used for the drug delivery in cancer, and their results indicate that functionalization with ligand can be used to actively target DNA nanocages to the cells overexpressing specific receptors followed by receptor-mediated endocytosis which optimize the cytotoxic effect of drugs [16]. Tao *et al.* developed the biodegradable alginate-chitosan hollow nanospheres (ACHNs) for the co-administration of doxorubicin and paclitaxel to human lung cancer A549 cells and their results indicate that the Dox-Ptx-ACHN co-delivery system efficiently inhibit cell proliferation and promote apoptosis and the synergistic effect on combined administration of doxorubicin and paclitaxel [17]. Chen *et al.* reported the Dox-loaded poly(lactic-*co*-glycolic acid) (PLGA)-polyethylene glycol (PEG)-folic acid (FA) nanoparticles. The internalization of Dox-loaded PLGA-PEG-FA nanoparticles was increased due to overexpressed folic acid receptors on cancer cell and subsequent receptor-mediated endocytosis leading to an enhanced uptake of nanocarrier for improved antitumor efficacy [18]. Muller *et al.* developed a nanostructured lipid carrier(s) which exhibited an improved loading capacity of drug and long-term stability. They constitute a highly concentrated however stable dispersions [19]. In the present study, we designed and developed a nanostructured lipid carrier(s) for combination chemotherapy to treat a resistant tumor. The folic acid conjugated paclitaxel-doxorubicin-loaded NLCs were prepared. Folic acid is a targeting moiety used to actively target the cancerous cells. The synergistic combination of paclitaxel and doxorubicin was used to treat resistant tumor. In addition, we characterized it for particle size and surface charges and morphology by using particle size analyzer (NanoPlus AT zeta/nanoparticle analyzer) and electron microscopy (FEI Tecnai G² F-20 S-Twin, Transmission electron microscope, Netherland). The formulations were evaluated for percent drug entrapment, *in vitro* drug release, and *ex vivo* cytotoxicity.

MATERIAL AND METHODS

Material

Distearoylphoshatidylethanolamine (DSPE) was obtained as a gift sample from lipoid (Germany); soyabean phosphatidylcholine, stearic acid, and cetyltrimethylammonium bromide were purchased from Sigma-Aldrich. Folic acid (FA) and *N*-hydroxysuccinimide (NHS) were purchased from Hi-Media. Dicyclohexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma Aldrich (India). All other reagents and solvents used were of analytical or HPLC grade.

Methods

Synthesis of Folate-DSPE Conjugates

Folate-DSPE was synthesized in two steps: step I involved the synthesis of NHS-ester of folic acid which was subsequently conjugated to activated DSPE in step II.

Step I: Synthesis of *N*-hydroxysuccinimide ester of Folic acid-*N*-hydroxysuccinimide ester of folic acid (NHS-folate) was prepared according to method reported by Lee *et al.* [20]. Briefly, folic acid (1 g) was dissolved in 20 ml of DMSO and 0.5 ml of TEA. Then, 0.94 g of DCC was accurately weighed and added to the solution and stirred for 1 h at room temperature under dark condition. Subsequently, 0.52 g of NHS was added to the mixture that was again stirred overnight under the same conditions. The byproduct dicyclohexyl urea (DCU) was removed by using centrifugation followed by filtration. The DMSO solution was then concentrated under reduced pressure, and NHS-folate conjugate so obtained was washed several times with anhydrous ether, dried under vacuum, and stored as yellow powder. The scheme of NHS-ester of folic acid synthesis discussed in step I is shown in Fig. 1a.

Step II: Coupling of carboxyl end of NHS-ester of folic acid with amine group of 1, 2 distearoylphosphatidyl ethanolamine (DSPE)—The folic acid was covalently coupled through its carboxyl group to the amino group of DSPE using EDC as a coupling agent. An equimolar amount of the above synthesized *N*-hydroxysuccinimide ester of folic acid and DSPE was dissolved in a common solvent. EDC was added to the folic acid DSPE solution and stirred continuously for 4 h at room temperature. Excessive amount of folic acid was removed by dialysis using a membrane with MW 2000 Da cut off and stored at 4°C. The conjugation scheme of NHS-folate with DSPE synthesis discussed in step II is shown in Fig. 1b. Conjugation was confirmed by using IR spectroscopy and H^1 NMR spectroscopy (Fig. 2a, b).

Preparation of Nanostructured Lipid Carrier(s)

Emulsion-evaporation method reported by Di *et al.* was used in the present work for the preparation of nanostructured lipid carrier(s) [21]. Briefly, doxorubicin hydrochloride (200 mg) was weighed and dissolved to form an aqueous solution using freshly prepared distilled water (10 ml) then triethylamine (1 ml) and acetone (3 ml) were added to the aqueous solution of doxorubicin hydrochloride followed by
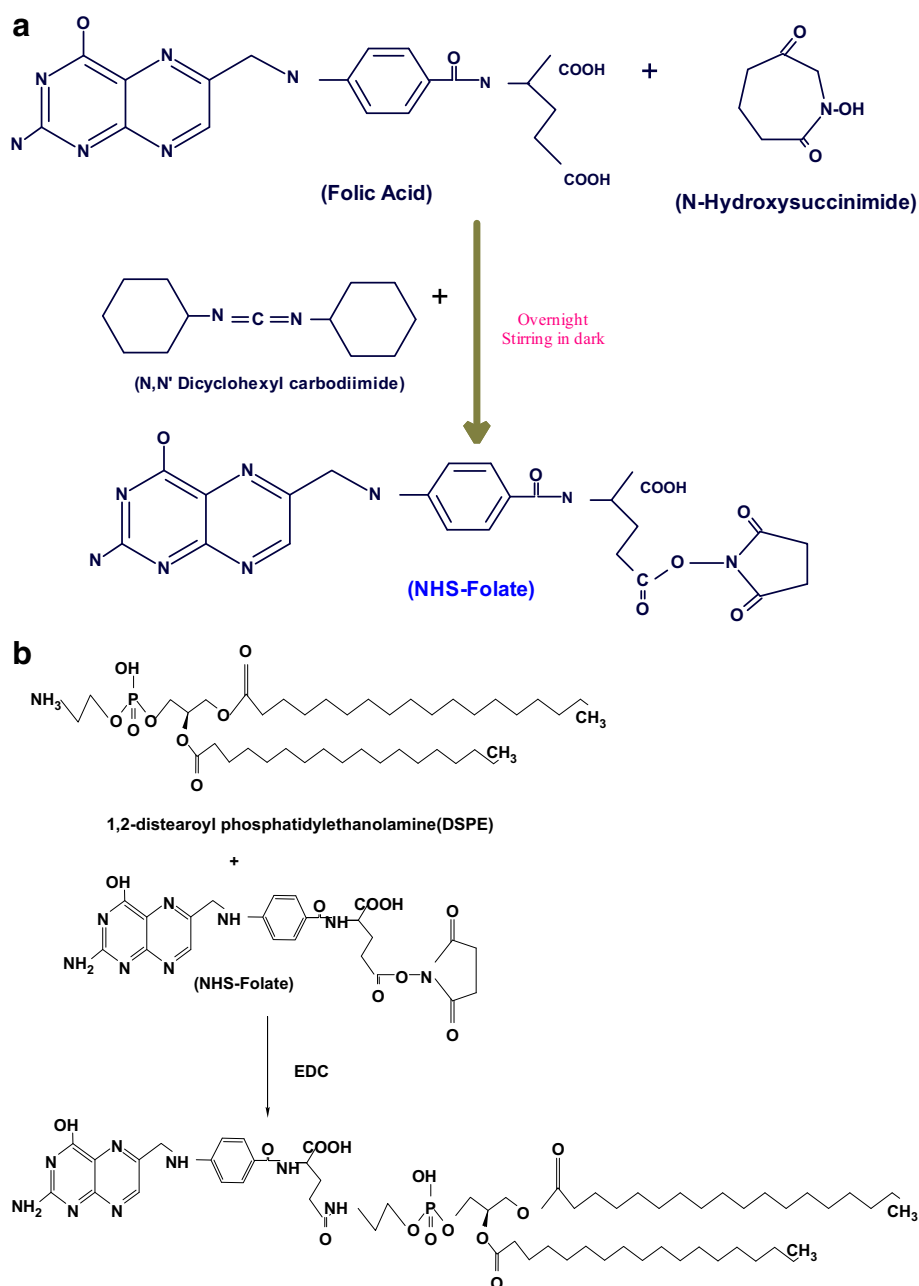
Fig. 1. **a** Activation of folic acid. **b** Conjugation of NHS-folate with DSPE

stirring over 24 h to get the doxorubicin base. Doxorubicin as base was dissolved in aqueous cetyltrimethylammonium bromide solution (100 ml, containing 0.5% cetyltrimethylammonium bromide) and heated by using a water bath maintained at 70 °C. Simultaneously, in another beaker paclitaxel (100 mg), stearic acid (500 mg) and soyabean phosphatidylcholine (500 mg) were dissolved into a mixture of acetone (5 ml) and ethanol (5 ml), *i.e.*, 1:1 (*v/v*), using a water bath maintained at 70 °C under constant stirring (1000 rpm, 1 h). The lipid(s) solution was injected into hot cetyltrimethylammonium bromide solution (0.5% *w/v*) to form an o/w nanoemulsion. The formed o/w nanoemulsion was then dispersed into cold distilled water (0°C) to form NLCs. By using the same method as mentioned above, the folic acid-conjugated doxorubicin-paclitaxel nanostructured

lipid carrier(s) were prepared by using DSPE as a co-lipid with soyabean phosphatidylcholine, where the DSPE is conjugated with folic acid.

PHYSIOCHEMICAL CHARACTERIZATION

Drug-Drug Compatibility

Infrared Spectroscopy

Drugs paclitaxel (5 mg) and doxorubicin hydrochloride (5 mg) were mixed with potassium bromide (100 mg) and compressed as pellets, for FTIR analysis to obtain spectra (Bruker Tensor-37, FTIR).
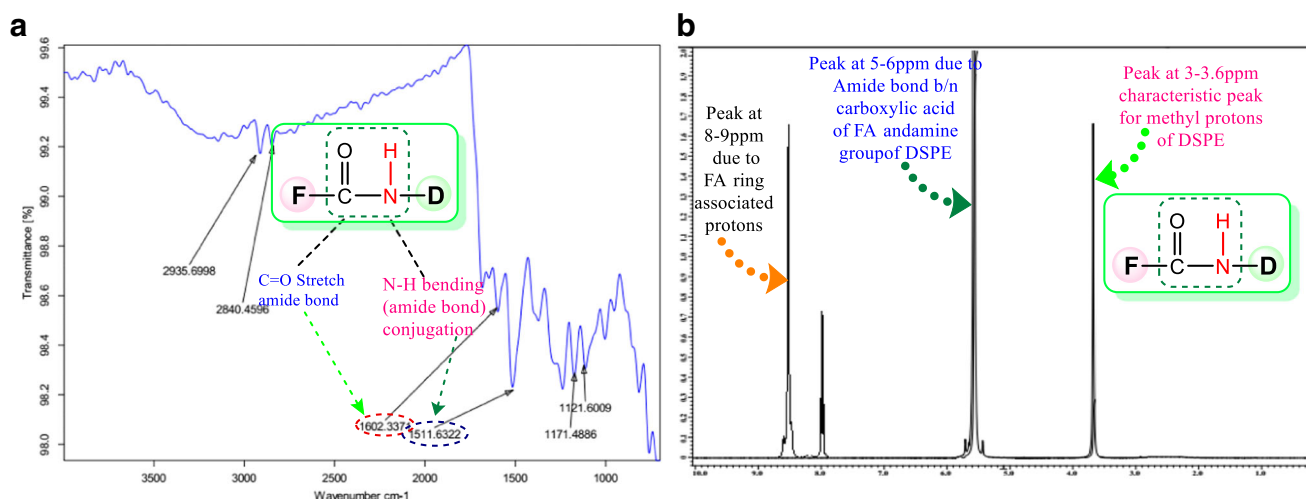
Fig. 2. a FTIR and **b** ^{1}H NMR spectrum of NHS-Folate DSPE conjugates

Differential Scanning Calorimetry

Differential scanning calorimetry analysis of drugs, *i.e.*, paclitaxel and doxorubicin hydrochloride as well as a physical mixture of both the drugs (1:1), was conducted over a temperature range between 0 and 250°C by using a differential thermal analyzer (NETZSCH STA 449 F1, Leading Thermal Analysis).

Surface Charge, Vesicle Size, and Size Distribution

The PDI and particle size of NLC dispersion in phosphate buffer (pH 7.4) were determined using zeta sizer (NanoPlus AT zeta/nanoparticle analyzer). The zeta potential of NLCs, dispersed in de-ionized sterile water at 25°C was assessed. All measurements were recorded in triplicate.

Transmission Electron Microscopy

NLCs were observed under transmission electron microscope (FEI Tecnai G^2 F-20 S-Twin, transmission electron microscope, Netherland). A drop of the formulation was placed on to a carbon-coated copper grid to form a thin film over the grid, and it was negatively stained with 1% phosphotungastic acid (PTA) before drying [22]. The grid was then allowed to be air dried at room temperature, and formulations were viewed under a transmission electron microscope and photomicrographs were captured at suitable magnification.

% Entrapment Efficiency (% EE)

The doxorubicin and paclitaxel contents in nanostructured lipid carrier(s) were determined by using UV-Visible (Shimadzu, Kyoto, Japan) and HPLC (Shimadzu Prominence, Japan) methods respectively [23]. The doxorubicin-paclitaxel-nanostructured lipid carrier(s) (D-P-NLCs) and FA-conjugated D-P-NLCs were centrifuged (1000×*g*) at 4°C for half an hour by using ultracentrifuge (HITACHI himac CP 100MX, Prepartive, Ultracentrifuge). Then, by using membrane filter with 0.45 μm pore size, the supernatant was filtered and then evaluated by using UV-Visible and HPLC for the measurement of entrapment efficiency of doxorubicin and paclitaxel, respectively.

The following equation was used to calculate the percentage entrapment efficiency:

$$\%EE = (Concentration\ of\ total\ drug - Concentration\ of\ free\ drug) \times Concentration\ of\ total\ drug^{-1} \times 100.$$

***In Vitro* Drug Release**

The *in vitro* drug release rate of doxorubicin (DOX) and paclitaxel (PTX) from NLCs was studied in PBS (pH 7.4 and 4.0) by using dialysis method [24, 25]. Briefly, the NLCs were suspended in 5 mL of the PBS (pH 7.4 and 4.0), transferred into a dialysis bag (MWCO 3500 Da), while the PBS (pH 7.4 and 4.0)/methanol (7:3) (*v/v*) was used as a dissolution medium. The dialysis bag(s) containing 5 ml of NLCs dispersion in PBS (pH 7.4) were placed in 45 mL of PBS (pH 7.4 and 4.0)/methanol (7:3) (*v/v*) under continuous shaking at 100 rpm at 37°C. Five milliliters of that solution was withdrawn periodically at schedule time and replaced with an equal volume of fresh PBS (pH 7.4 or 4)/methanol (7:3) (*v/v*). The amount of DOX released was determined using UV-Vis spectrometer (Shimadzu, Kyoto, Japan); amount of PTX released was measured by HPLC (Shimadzu Prominence, Japan) (*n* = 3) [17, 23, 26].

***Ex Vivo* Cytotoxicity Study**

The MCF-7/ADR cells were treated with folic acid-doxorubicin-paclitaxel-nanostructured lipid carrier(s) (FA-D-P-NLCs), D-P-NLCs, plain (without drug loading)-nanostructured lipid carrier(s) (B-NLCs), DOX and PTX mixed solution (D-P-sol), doxorubicin solution (D-sol), paclitaxel solution (P-sol), and untreated MCF-7/ADR cells were used as positive control [27]. Dulbecco's modified Eagle medium (0.2 ml) containing 10% fetal bovine serum (FBS) was placed in a 48-well plate then cells were seeded into each well. Under the same conditions, after overnight incubation, each well was refreshed with 0.2 ml serum free medium (SFM) on an alternative day. The cells were then treated with serum-free medium (0.2 ml) comprising of various concentrations of

nanostructured lipid carrier(s). Subsequently, the cells were treated with 0.2 ml SFM comprising of various concentrations of NLCs. The MTT reagent was used to determine the cell viability by using plate a reader; the absorbance was measured at λmax 590 nm. The relative viability was calculated as the absorbance at 590 nm of treated MCF-7/ADR cells divided by the absorbance at 590 nm of the untreated MCF-7/ADR cells. The concentration of drug causing 50% growth inhibition (GI_{50}) was calculated using social science version 17 software [21].

Statistical Analysis

The study data collected as an average of three readings were analyzed statistically. All the results were reported as mean ± SD (standard deviation). By applying one-way ANOVA, the statistical significance was determined. The differences between experimental groups when the P value was less than 0.05 ($P < 0.05$) were considered significant.

RESULT AND DISCUSSION

Drug-Drug Compatibility

Infrared Spectroscopy and Nuclear Magnetic Resonance

The specific signal of (C–O) stretch of secondary alcohol at 1143 cm^{-1}, typical signal of out-of-plane O–H band at 650 cm^{-1} and characteristics absorption bands of C=O stretch of ester and acid at 1735 and 1710 cm^{-1} respectively, confirmed the presence of paclitaxel (Fig. 3a). Similarly, the typical signal of C=O stretch of ketone at 1730 cm^{-1}, signal of C=C stretch of benzene at 1510 cm^{-1}, and signal at 1245 and 704 cm^{-1} for C–O–C asymmetric stretching and out-of-plane
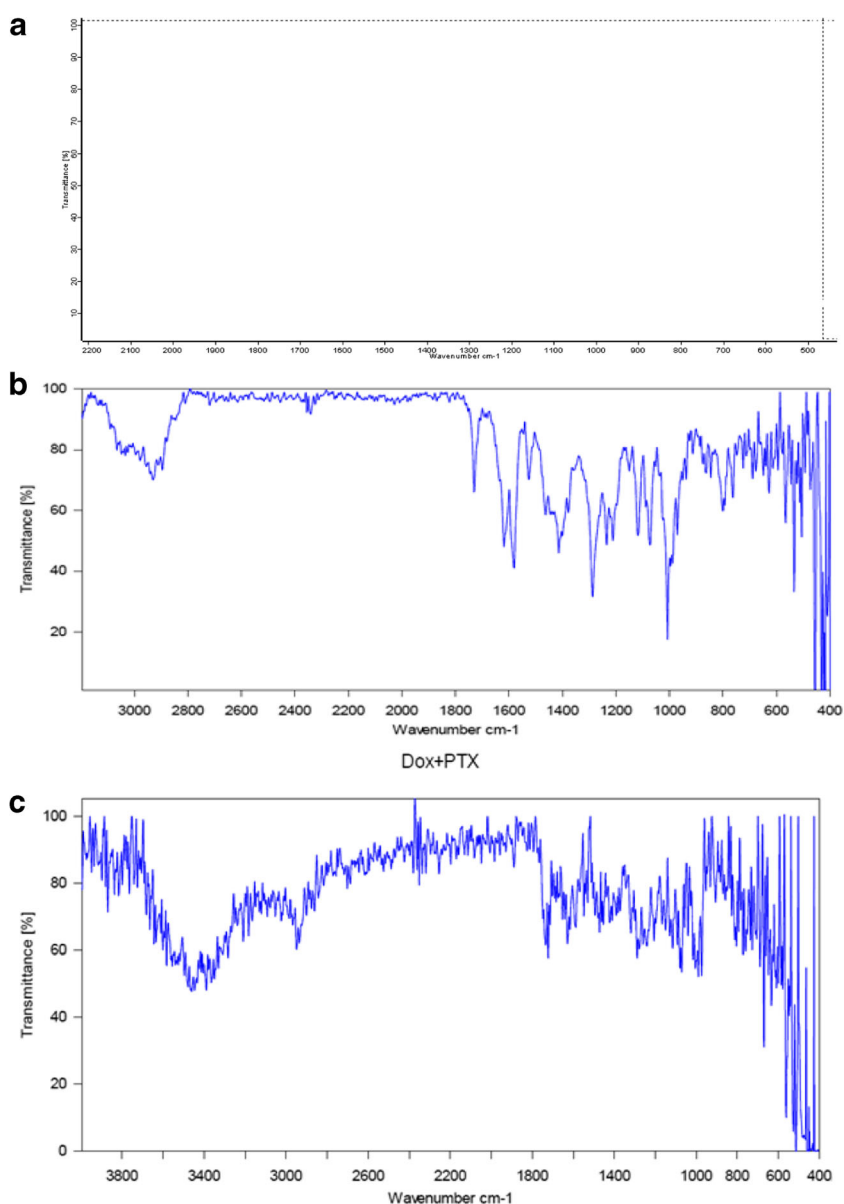

Fig. 3. **a** FTIR of paclitaxel. **b** FTIR of doxorubicin hydrochloride. **c** FTIR of paclitaxel + doxorubicin hydrochloride (mixture)
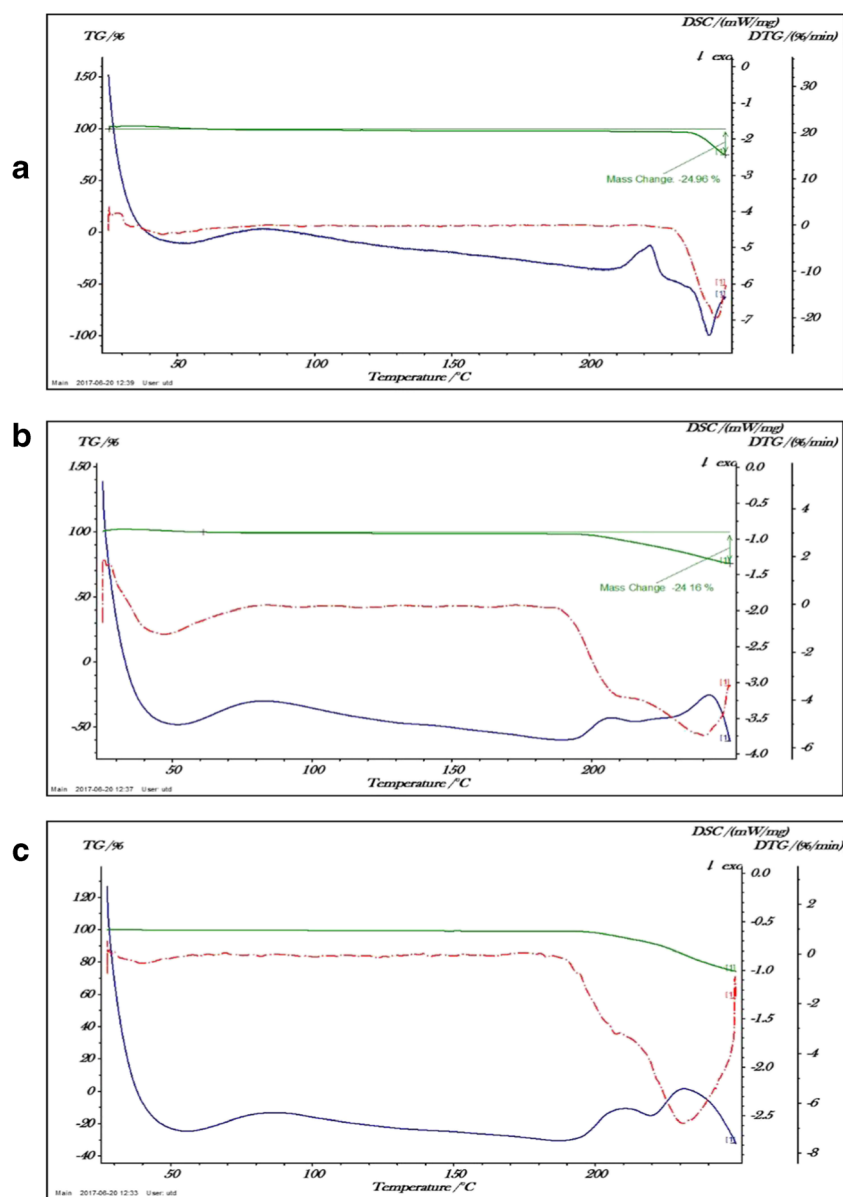
Fig. 4. DSC graph of **a** paclitaxel, **b** doxorubicin hydrochloride, and **c** paclitaxel/doxorubicin hydrochloride (1:1)

N–H wagging respectively [28] confirmed the presence of doxorubicin hydrochloride (Fig. 3b). Figure 5a–c shows both the standard spectra of paclitaxel, doxorubicin hydrochloride, and mixture of paclitaxel and doxorubicin hydrochloride which also confirm the compatibility of the drugs with no interaction between the drugs or excipients.

Differential Scanning Calorimetry

The characteristics DSC curve obtained for the drugs (paclitaxel and doxorubicin hydrochloride) showed their characteristic endothermic peaks above 180°C temperature. DSC curve for mixture of both the drugs, *i.e.*, paclitaxel and doxorubicin hydrochloride (1:1), showed their characteristic endothermic peaks above 180°C temperature when both the drugs were taken in a mixture. TGA curve obtained for drugs, *i.e.*, paclitaxel and doxorubicin hydrochloride, showed

their characteristic mass loss was about 24.96 and 24.16% above 200°C temperature, respectively. TGA curve for mixture of both the drugs, *i.e.*, paclitaxel and doxorubicin hydrochloride (1:1), showed their characteristic mass loss was about 24% above 200°C temperature. Figure 4a–c shows characteristic DSC and TGA curve for paclitaxel, doxorubicin hydrochloride, and mixture of paclitaxel and doxorubicin hydrochloride revealing no interaction indicating the compatibility between the drugs.

Folate-DSPE Conjugates

The final conjugates were synthesized by forming amide bond between carboxylic group of folic acid and mine group of DSPE. Folate-DSPE conjugates were characterized by FTIR and H[1] NMR spectroscopy (Fig. 2a, b). A typical signal of amide stretch (C=O) absorption band at 1603 cm^{−1} and

Table I. Optimized Formulation Variables and Value of Size, Zeta Potential, and PDI for NLCs

Formulation	Amount of lipid (mg)	Stearic acid content (mg)	Cetrimide con. (%w/v)	Lipid/ DSPE ratio	Stirring speed (rpm)	Stirring time (min)	Particle size (nm)	PDI	Zeta potential (mV)
D-P NLCs	500	500	0.5	9:1	1000	60	184 ± 5.7	0.180 ± 0.07	+16.9 ± 0.4
FA-conjugated D-P NLCs	500	500	0.5	9:1	1000	60	196 ± 2.5	0.214 ± 0.04	+23.4 ± 0.3

Results are represented as mean ± SD (n = 3)

amide banding (N–H) at 1511 cm^{-1}, confirmed the synthesis of NHS folate-DSPE conjugates (Fig. 2a). Conjugates were further characterized by H^1 NMR, and the characteristic peaks of methyl protons of DSPE are recorded at about 3–3.6 ppm. The proton peaks at 5-6 ppm confirmed the formation of amide bond between the carboxylic group of folic acid and the amine group of DSPE and the characteristic peaks of folic acid ring-associated protons recorded at about 8-9 ppm; H^1 NMR confirmed the synthesis of NHS folate-DSPE conjugates (Fig. 2b).

Nanostructured Lipid Carrier(s)

Zeta potential, PDI, and particle size of doxorubicin-paclitaxel-nanostructured lipid carrier(s) (D-P-NLCs) and FA-conjugated D-P-NLCs (FA-D-P-NLCs) are summarized in Table I. The particle sizes of D-P-NLCs and FA-conjugated D-P-NLCs were found to be 184 ± 5.7 and 196 ± 2.5 nm, respectively. The average NLC size recorded for different formulation was in a range of 182 to 200 nm. Figure 5a, b shows the size distribution curve of D-P-NLCs and FA-D-P-
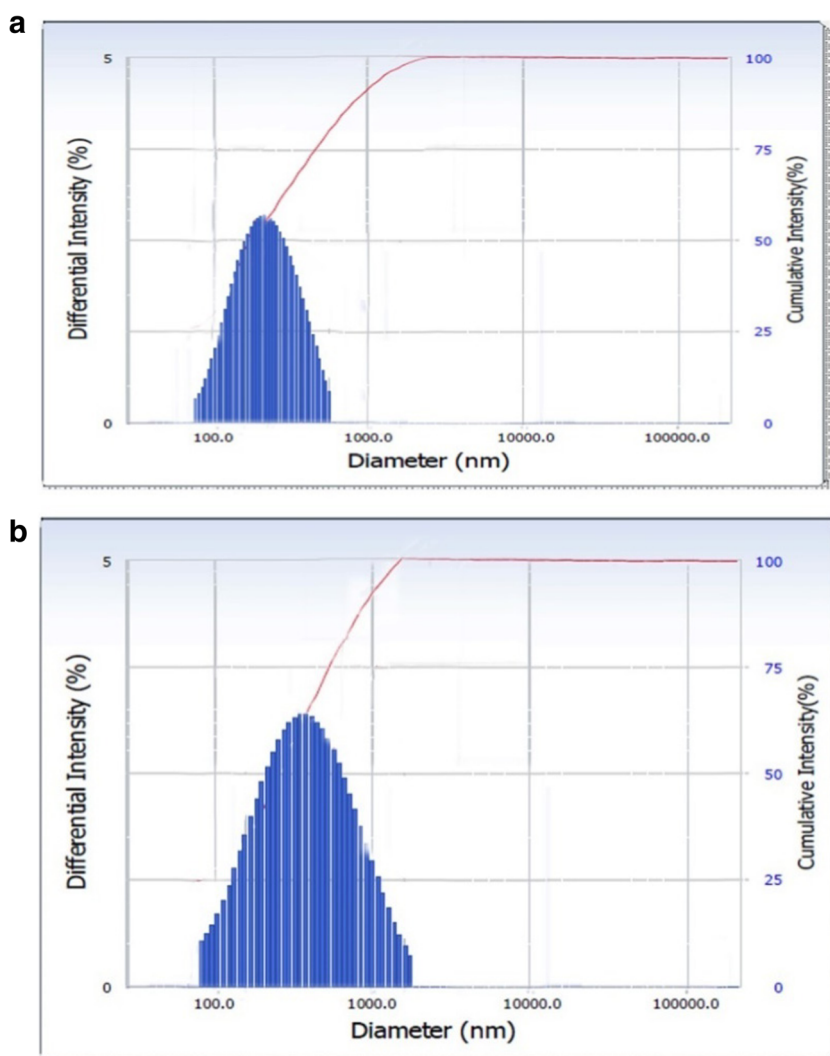

Fig. 5. Size distribution curve of **a** doxorubicin-paclitaxel-nanostructured lipid carrier(s) (D-P-NLCs) and **b** FA-conjugated D-P-NLCs (FA-D-P-NLCs)
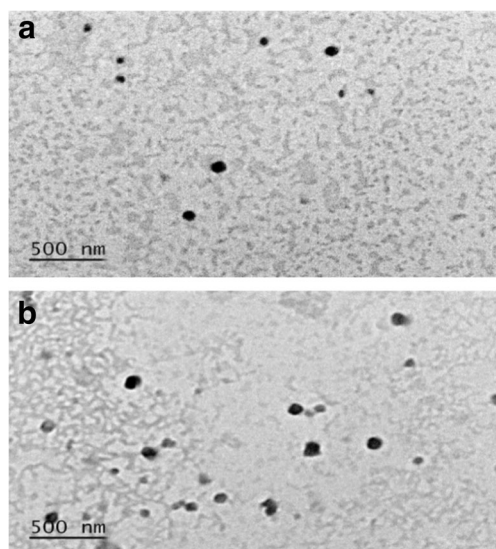
Fig. 6. TEM photomicrograph of **a** doxorubicin-paclitaxel-nanostructured lipid carrier(s) (D-P-NLCs) and **b** FA-conjugated D-P-NLCs (FA-D-P-NLCs)

NLCs, respectively. The average size of nanocarriers and its effect on *in vitro* cytotoxicity, as well as sustained blood circulation and enhanced bioavailability, has been recognized [29, 30]. Cellular uptake of nanoparticles is affected by surface charge of nanoparticles because of the electrostatic interactions between the nanoparticles and cell surface [31]. The

EEs are between 84 and 89%. Zeta potentials recorded were invariably positive. The zeta potential of NLCs is positive owing to the presence of cationic surfactant cetrimide. The nanocarriers possessing positive charge may facilitate their efficient cellular interaction and uptake through endocytosis [32]. It could have proceeded through bio cell-associated negative charge, prone for interacting with cationic charge-bearing composites, resulting into cell penetration and subsequent internalization [33]. The cationic charge on NLC(s) may lead to endosomal destabilization resulting into burst release of both drugs (paclitaxel and doxorubicin) in the cytosol.

Transmission Electron Microscopy

Prepared D-P-NLCs and FA-D-P-NLCs were observed under transmission electron microscope (FEI Tecnai G^2 F-20 S-Twin, transmission electron microscope, Netherland). The transmission electron microscopy (TEM) studies revealed smooth and a nearly spherical shape of D-P-NLCs and FA-D-P-NLCs. The negative staining of samples provides a useful approach for the high resolution of nanoformulation. It was evident from TEM studies that the developed nanocarrier systems were of nanometric size range. D-P-NLCs and FA-D-P-NLCs were viewed under a transmission electron microscope, and photomicrographs were captured at suitable magnification. The photomicrographs are shown in Fig. 6a, b.
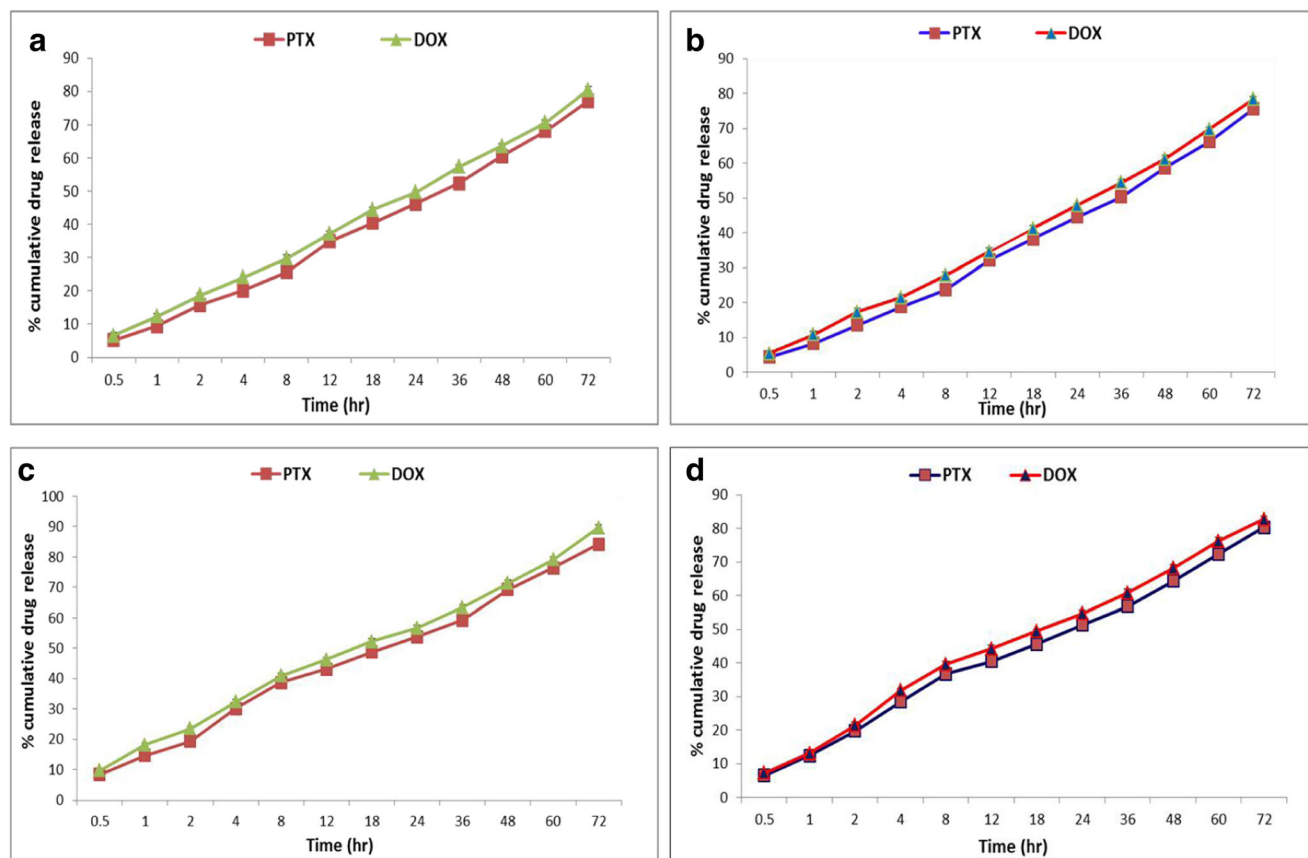

Fig. 7. % Cumulative PTX and DOX release in PBS (pH 7.4)/methanol (7:3) from **a** NLCs, **b** FR-conjugated NLCs, **c** NLCs in PBS (pH 4)/methanol [7:3], and **d** FR-conjugated NLCs in PBS (pH 4)/methanol [7:3]

In Vitro Release Assay

Formulation of paclitaxel (PTX) with doxorubicin (DOX)-loaded NLCs showed cumulative drug release 77.2 and 80.5% for paclitaxel and doxorubicin respectively in 72 h in PBS (pH 7.4)/methanol (7:3). The release pattern is graphically shown in Fig. 7a, while folic acid-conjugated PTX with DOX-loaded NLCs showed 75.6 and 78.4% cumulative release of paclitaxel and doxorubicin respectively in 72 h in PBS (pH 7.4)/methanol (7:3) as shown graphically in Fig. 7b, indicating relatively slower release of drugs in case of folate-conjugated NLCs compared to unconjugated NLCs at pH 7.4. Further, the cumulative drug release was also studied at pH 4 as well, and the formulations of PTX with DOX-loaded NLCs showed cumulative drug release 82.5 and 85.6% of paclitaxel and doxorubicin, respectively, in 72 h in PBS (pH 4.0)/methanol (7:3) as shown graphically in Fig. 7c, while folic acid-conjugated PTX with DOX-loaded NLCs showed cumulative drug release 80.4 and 82.8% of paclitaxel and doxorubicin, respectively, in 72 h In PBS (pH 4.0)/methanol (7:3) as shown graphically in Fig. 7d. The

results suggest relatively slower release of drugs in case of conjugated NLCs as compared to unconjugated NLCs. The results further indicate that the NLCs conjugated with folic acid mitigated the release of drug by constituting secondary barrier. Nevertheless, the drug(s) release in PBS at pH 4 was faster as compared to pH 7.4, suggesting a pH-modulated drug(s) release, which would be facilitated by the acidic bioenvironment of tumor or in the endosomes especially in the case of targeted drug delivery.

Ex Vivo Cytotoxicity Study

The relative ability of FA-D-P-NLCs, D-P-NLCs, plain (without drug loading)-nanostructured lipid carrier(s) (B-NLCs), DOX, and PTX mixed solution (D-P-sol), doxorubicin solution (D-sol), paclitaxel solution (P-sol) to inhibit MCF-7/ADR cells was estimated by using MTT assay. The cells which are treated with the plain NLCs show no appreciable cell inhibition effect as compared to untreated cells indicating that the nanocarrier does not have any pharmacodynamic effect.
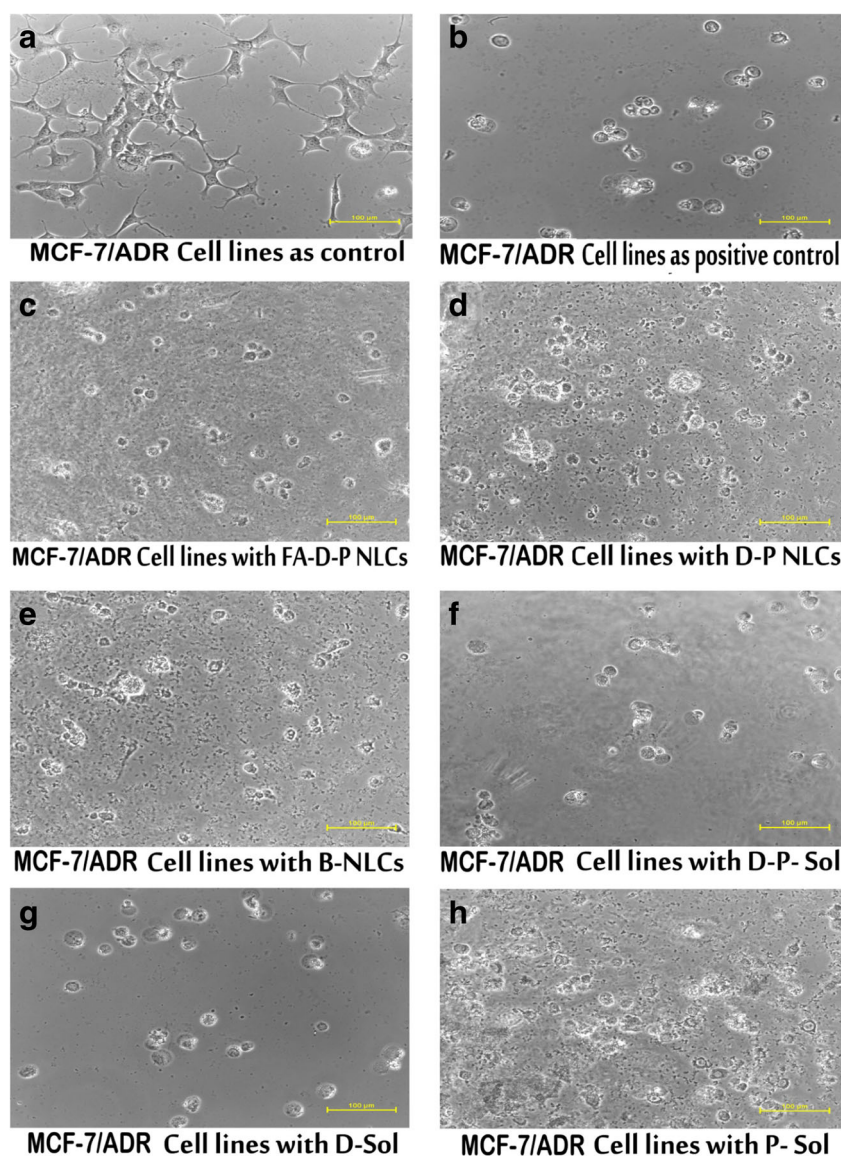

Fig. 8. Photomicrograph of MCF-7/ADR cell lines (control and formulation treated)

MTT assay was performed on MCF-7/ADR cell lines and photomicrographs of MCF-7/ADR cell lines (control and formulation treated) are shown in Fig. 8. The GI_{50} of FA-D-P-NLCs for MCF-7/ADR cells was measured to be 1.04 μg/ml, which is three times less than the GI_{50} value (3.12 μg/ml) of D-P-NLCs. The GI_{50} of DOX solution, PTX solution, and mixture of DOX-PTX solution recorded was 5.76 μg/ml, 4.82 μg/ml, and 3.89 μg/ml, respectively. The maximum cell-specific cytotoxicity was thus recorded in the case of system which carried and delivered the drugs in combination to the target cells, *i.e.*, FA-D-P-NLCs. Further, the cytotoxicity exhibited by D-P-NLCs was relatively more as compared to DOX-PTX mixed solution which could be attributed to relatively higher drug accumulation that resulted due to cationic carrier(s) and negatively charged MCF-7/ADR cell line interaction as compared to plain drug(s) solution. Nevertheless, the activity of D-P-NLCs was less than FA-D-P-NLCs. This differentiated activity profile may be ascribed to the targetability of FA-D-P-NLCs leading to cell-specific higher accumulation and hence in turn the activity. From the results, it is evident that the amount of each drug could be reduced significantly on account of co-administration of two drugs by using target-oriented NLCs conjugated to folic acid. The targeting moiety helps localize both the drugs in the vicinity of the cancer cells followed by internalization finally resulting into better therapeutic effect with reduced possibility of systemic toxicity.

CONCLUSION

In a promising study, we are reporting a combination chemotherapy based on paclitaxel and doxorubicin co-entrapped within a single nanostructured lipid carrier(s). Established formulations were characterized for particle size and surface characteristics by a Zetasizer and electron microscopy, entrapment efficacy, *in vitro* drug release, and cytotoxicity studies. From the results of the studies, it can be concluded that folic acid-conjugated PTX with DOX-loaded NLCs possess better *in vitro*, *ex vivo*, and pharmacodynamic profile as compared to unconjugated NLCs. Folic acid-doxorubicin-paclitaxel-nanostructured lipid carrier(s) however shows the maximum selective cytotoxicity and synergistic effect of drug combination in tumor cells *in vitro*. Lastly, we conclude that the combination chemotherapy of paclitaxel and doxorubicin contained within a single nanostructured lipid carrier with folic acid as a targeting moiety has enormous potential for development of targeted combination chemotherapy based on the present system.

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest The authors declare that they have no conflict of interest.

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