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Anti-Cancer Activity of Doxorubicin-Loaded Liposomes Co-Modified with Transferrin and Folic Acid

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Abstract

Cancer-specific drug delivery represents an attractive approach to prevent undesirable side-effects and increase the accumulation of the drug in the tumor. Surface modification of nanoparticles such as liposomes with targeting moieties specific to the up-regulated receptors on the surface of tumor cells thus represents an effective strategy. Furthermore, since this receptor expression can be heterogeneous, using a dual-combination of targeting moieties may prove advantageous. With this in mind, the anti-cancer activity of PEGylated doxorubicin-loaded liposomes targeted with folic acid (F), transferrin (Tf) or both (F+Tf) was evaluated. The dual-targeted liposomes showed a 7-fold increase in cell association compared to either of the single-ligand targeted ones in human cervical carcinoma (HeLa) cell monolayers. The increased penetration and cell association of the dual-targeted liposomes was also demonstrated using HeLa cell spheroids. The *in vitro* cytotoxicity of the doxorubicin liposomes (LD) was then evaluated using HeLa and A2780-ADR ovarian carcinoma cell monolayers. In both these cell lines, the (F+Tf) LD showed significantly higher cytotoxic effects than the untargeted, or single-ligand targeted liposomes. In a HeLa xenograft model in nude mice, compared to the untreated group, though the untargeted LD showed 42% tumor growth inhibition, both the (F) LD as well as (F+Tf) LD showed 75% and 79% tumor growth inhibition respectively. These results thus highlight that though the dual-targeted liposomes represent an effective cytotoxic formulation in the *in vitro* setting, they were equally effective as the folic acid-targeted liposomes in reducing tumor burden in the more complex *in vivo* setting in this particular model.

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

Supporting Information. Evaluation of ligand density for cell association on HeLa cells in folic acid deficient media.

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Graphical Abstract

Keywords

Liposomes; dual-targeting; nanoparticles; doxorubicin; cancer; HeLa; xenograft; folic acid; transferrin; A2780-ADR; receptor targeting; nanomedicine

INTRODUCTION

Cancer is still one of the leading causes of death worldwide and arises when a normal cell undergoes a series of genetic mutations resulting in its uncontrolled cell growth and proliferation, causing malignancy. Though the fight against cancer has been long-standing, mortality rates for a variety of cancer types have only decreased by less than 2%.¹ The current standard of care for solid tumors comprises of debulking surgery followed by treatment with chemotherapeutic drugs. Since the conceptualization of the “magic bullet” concept, the development of target-specific drugs has always been widely pursued.² Although a number of novel anticancer drugs have been developed, the use of existing drugs such as doxorubicin (Dox), paclitaxel and cisplatin are still predominant in the clinic.³ More specifically, anthracycline drugs such as Dox are considered one of the most effective anticancer drugs ever developed.⁴ It has been shown that Dox induces caspase-dependent apoptosis in cancer cells due to oxidative DNA damage in addition to topoisomerase II inhibition.^{5, 6} However, its use has been considered a double edged sword as though it is a potent anticancer molecule, it is also known to target other healthy tissues in the body resulting in toxicity to vital organs such as the heart.

The use of nanoparticles such as liposomes represents an efficient strategy to mitigate these effects by allowing for the increased accumulation of drugs at the target site while at the same time minimizing drug interaction with healthy cells. Since the conceptualization of their use as drug carriers in the 1970s, liposomes have made a lot of progress being constantly streamlined and have become the carrier of choice for the delivery of a number of promiscuous drug candidates.⁷⁻⁹ PEGylation of these nanoparticles is known to impart “stealth” properties to liposomes allowing longevity by screening the adsorption of proteins

onto their surface thereby minimizing uptake by the mononuclear phagocyte system (MPS).^{10–12} In addition, being in the nanometer size range allows them to accumulate preferentially into tumor tissues by the enhanced permeability and retention (EPR) effect.¹³

In order to enhance their cell-specific as well as intracellular delivery, liposomes can be further modified with targeting ligands in order to exploit tumor characteristics since cancer cells are known to over-express a number of cell surface proteins such as integrins, growth factor receptors, glucose transporters, folate receptors (FRs) and transferrin receptors (TfRs) amongst others.¹⁴

FR is a very well-studied protein in cancer therapy as folic acid plays a vital role in DNA synthesis to support the proliferation of cancer cells. While FR alpha (FR- α) is overexpressed in a variety of cancer types, normal tissues contain very low levels of the receptor and in some cases they are located on the apical side away from circulation such as in the kidney tubules and air sacs of the lungs, thereby minimizing the potential for off-target toxicities.^{15, 16} As a result, a number of macromolecular drugs as well as nanoparticles have been targeted with folic acid to enhance their antitumor efficacies both *in vitro* and *in vivo*.^{17,18, 19} Similarly, the TfR has been widely exploited for drug delivery into cancer cells as it is overexpressed in many cancer types due to its role in iron homeostasis and cell proliferation as well as its receptor mediated endocytosis.²⁰ For example, Tf-targeted liposomes have been developed for the delivery of anticancer drugs such as oxaliplatin, doxorubicin, 5-fluorouracil, ceramide as well as DNA and siRNA.^{21–26}

In order to further explore the concept of targeting, the concept of ‘dual-targeting’ was pursued, which employs the use of two different target-specific ligands on the surface of the same nanocarrier to allow for synergistic effects. Inspired by the strategy employed by natural viruses, it may be advantageous to develop a combination of ligands that can interact with the various cell-surface antigens in order to overcome the heterogeneous pattern of expression in various tumor types and increase the number of potential binding sites for nanomedicines on the surface of tumor cells and within the tumor.^{27, 28} The different dual-targeted strategies reported recently, comprise of targeting with cell penetrating peptides (CPPs) and hyaluronic acid²⁹, anti-myosin antibody and TAT peptides³⁰, α CD19 and α CD20 antibodies³¹, as well as targeting the FR and the epidermal growth factor receptor³².

Though in general the use of targeting has proven advantageous to the delivery of drugs, there have been many instances where there has not been a significant improvement in antitumor efficacy *in vivo* due to the heterogeneous nature of cancer^{22, 33}. The *in vivo* setting is fairly complex and there are a number of barriers to the delivery of nanoparticles.³⁴ Therefore we decided to investigate if targeting would allow for better cell association and antitumor efficacy *in vitro* and *in vivo*, as well as to ascertain if there was a good correlation among the three experimental models for the various targeted groups. PEGylated Dox-loaded liposomes targeted with folic acid, Tf as well as both were developed and their anticancer potential was then evaluated *in vitro* in cancer cell monolayers and spheroids as well as *in vivo*.

EXPERIMENTAL SECTION

Materials, Cell Culture and Animals

FITC-labeled mouse monoclonal anti-transferrin receptor antibody (ab47095) was purchased from Abcam (San Francisco, CA); goat anti-folate receptor alpha primary antibody (sc-16386), FITC-labeled secondary donkey anti-goat antibody (sc-2024) and FITC-labeled normal mouse IgG (sc-2855) (as negative control) were purchased from Santa Cruz Biotechnology (Dallas, TX); nitrophenylcarbonyl-PEG₃₄₀₀-nitrophenylcarbonyl (NPC-PEG-NPC) and amino-PEG₃₄₀₀-DSPE were from Laysan Bio (Arab, AL); eggphosphatidylcholine (ePC), cholesterol, cholesteryl hemisuccinate (CHEMS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG), (lissamine rhodamine) DPPE (rhodamine) were from Avanti (Alabaster, AL); Dragendorff's reagent, Molybdenum blue, triethylamine (TEA), Sepharose CL-4B (40–165 μ m), transferrin, folic acid, dicyclohexylcarbodiimide (DCC) and Ninhydrin were from Sigma-Aldrich (St. Louis, MO); microBCA[™] protein assay kit, Whatman nucleopore polycarbonate membranes 19 mm at 0.2 μ m, 0.1 μ m and 0.05 μ m pore sizes, bovine serum albumin (BSA) and agarose were from Fisher/Thermo Scientific (Waltham, MA); Spectra/Por pre-wetted 300,000 MWCO dialysis membranes and Spectra/Por 12–14 kDa MWCO dry membranes were from Spectrum Labs (Rancho Dominguez, CA); CellTiter-Blue[®] cell viability assay was from Promega Corporation (Madison, WI); doxorubicin hydrochloride was from LC Labs (Woburn, MA) and Lancix Chemicals (Shanghai, China).

Streptomycin (25 μ g/mL)/Penicillin (10,000 U/mL) solution, RPMI media, Cellstripper[™] as well as Trypsin/EDTA were purchased from Corning/Mediatech (Manassas, VA); RPMI without folic acid was purchased from Life Technologies (Carlsbad, CA); F-12K media, normal human skin fibroblasts (CCD-27Sk), human cervical cancer cells (HeLa) and human umbilical vein endothelial cells (HUVEC) were from ATCC (Manassas, VA); fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA); adriamycin-resistant human ovarian carcinoma cells (A2780-ADR) were purchased from Sigma-Aldrich. The HeLa and A2780-ADR cells were grown in RPMI media. Additionally, HeLa were also grown in folic acid deficient RPMI for 2 weeks for the cell association experiments. HUVEC cells were grown in F-12K media supplemented with 0.1 mg/mL Heparin and 0.03 mg/mL endothelial cell growth supplement. All media were supplemented with 10% FBS and 1% penicillin-streptomycin solution. Cells were all grown at 37°C with 5% CO₂.

Matrigel[®] matrix used for tumor inoculation was purchased from Corning. 6–8 week old female NCR nude mice (Nu⁻/Nu⁻) were purchased from Taconic Biosciences (Rensselaer, NY).

Synthesis of pNP-PEG₃₄₀₀-DOPE

The pNP-PEG-DOPE was synthesized and purified by slight modifications to a previously established method.³⁵ 64.6 μ mol NPC-PEG₃₄₀₀-NPC (or pNP-PEG₃₄₀₀-pNP) was first dissolved in 1 mL chloroform and then reacted with 12.9 μ mol DOPE in the presence of 38.8

μmol TEA. The reaction was incubated overnight at room temperature (RT) in an argon atmosphere under constant stirring. The reaction was monitored using thin-layer chromatography (TLC) in a mixture of chloroform-methanol as an eluent at an 80:20 volumetric ratio. Dragendorff's reagent and Molybdenum blue were used to visualize the PEG and DOPE respectively. After completion of the reaction, the solvent was removed from the mixture using rotary evaporation followed by freeze drying on a FreeZone 4.5L Freeze Dry system (Labconco, Kansas City, MO). The product was then dissolved in 0.001 M HCl, pH 3.0 at a lipid concentration of 200 mg/mL and separated on a column loaded with Sepharose CL-4B and eluted with 0.001M HCl. The various fractions collected were then analyzed for the presence of the product by TLC. Only those fractions containing the product were then pooled together and freeze dried. This was dissolved in chloroform (at 5 mg/mL) and stored in -80°C until further use.

Synthesis of Transferrin-PEG₃₄₀₀-PE

A thin film containing 2 mg of pNP-PEG-DOPE was obtained by removing the chloroform by drying under a stream of nitrogen gas, followed by freeze drying for a minimum of 4 hours to remove trace solvents. The film was hydrated with 1mL of a 15mg/mL solution of Tf in phosphate buffered saline (PBS) pH 8.5. The reaction was incubated at RT overnight under constant stirring. Following this, the unreacted Tf was removed by dialyzing the reaction mixture against 2L of deionized (DI) water for 30 minutes followed by dialysis against 2L of PBS pH 7.4 for 4 hours, using an MWCO of 300,000 Da. The concentration of the product, Tf-PEG-DOPE was then determined using the microBCA™ assay kit.

Synthesis of Folic Acid-PEG₃₄₀₀-DSPE

The folic acid conjugate (FA-PEG-DSPE) was synthesized by slight modification of methods described previously.³⁶ Folic acid (4.52mg, 10.24 μmol) was added to 4mL of DMSO under constant stirring followed by 2mL pyridine. DCC (6.34mg, 30.76 μmol) was then added to the reaction solution and stirred at RT for 15 minutes. Amino-PEG₃₄₀₀-DSPE (20mg, 5.12 μmol) was then added to the above reaction mix. The reaction was then carried out at RT for 4 hours under constant stirring. Using TLC, disappearance of amino-PEG-DSPE from the reaction mix was confirmed by the Ninhydrin spray. The reaction mix was dried under nitrogen and then placed under a rotary evaporator for 1 hour to remove the pyridine. After addition of 8mL DI water, the solution was centrifuged at 5000 RPM for 15 minutes to remove trace insolubles. The supernatant was then dialyzed against water (5 × 2L) over 48 hours using an MWCO of 300,000 Da to ensure complete removal of free folic acid. The dialyzate was then freeze dried and dissolved in a 90:10 mixture of chloroform: methanol (1 mg/mL) and stored at -80°C until further use.

Preparation of Liposomes

A lipid film consisting of ePC, cholesterol, CHEMS and DOPE (66:24:5:5 mole %) (See Table 1) was obtained by removal of chloroform by rotary evaporation followed by freeze drying for a minimum of 4 hours. The film was then hydrated with 250 mM ammonium sulfate pH 7.0 so as to maintain a lipid concentration of 15 mg/mL. The solution was vortexed and then sequentially extruded through 200, 100 and 50 nm polycarbonate membranes. The liposomes were then dialyzed against PBS pH 7.4 using a MWCO of 12–

14kDa for 1 hour at RT to exchange the outer liposomal buffer. The liposomes were then incubated with Dox (5 mg/mL stock solution in 0.9% NaCl) at a Dox: lipid mole ratio of 1:5 for 1 hour at 65°C. Following this, the unencapsulated Dox if any, was removed by dialysis against PBS pH 7.4 using a MWCO membrane of 12–14 kDa for 4 hours at RT. The FA-PEG-DSPE, Tf-PEG-DOPE and PEG₂₀₀₀-DSPE (PEG) were then added at 0.5, 0.05 and 3 mole % respectively to the liposomes by the post-insertion method. Briefly, a thin film of the PEG and FA-PEG-DSPE obtained by the removal of organic solvent (by drying under a stream of nitrogen gas followed by freeze drying for a minimum of 4 hours), was hydrated with the Tf-PEG-DOPE solution or an equal volume of PBS to form mixed micelles. These were then added to the liposomal solution and incubated at 37°C overnight to allow for complete incorporation of the PEG chains into the liposome.

Characterization of Liposomes

Particle size and zeta potential analysis was carried out using an N4 Coulter particle size analyzer (West Lafayette, IN) and Zetaplus (Brookhaven Instruments Corporation, Holtsville, NY) respectively. For particle size analysis, 5µL of the liposomal solution was mixed with 990µL of 1mM KCl while for zeta potential, 50µL was mixed with 1.5mL of 1mM KCl. For the determination of liposomal Dox content, the liposomes were dissolved in methanol at a dilution factor of 50 and the absorbance was measured at 480 nm. The drug concentration was determined by comparison with a standard curve of free Dox in methanol (0–60 µg/mL). To characterize the pH-dependent release of Dox from the liposomes, 0.8 mL of the liposomal Dox solution was dialyzed against 40 mL of either 300mM citrate buffer pH 5.0 or PBS pH 7.4 using a MWCO membrane of 12–14kDa for 96 hours. The release samples were taken at the mentioned time points while being replaced with equal volumes of fresh buffer and the Dox content in the release buffer was determined using HPLC (Hitachi Elite LaChrom, Pleasanton, CA). For the HPLC method, a Waters (Milford, MA) Cortecs™ C18 column (2.7 µm, 4.6 mm × 150 mm) was used with a mobile phase of 60% (v/v) acetonitrile and 40% (v/v) of 0.1% trifluoroacetic acid in water with a flow rate of 1 mL/min. The Dox peak was determined by fluorescence (Ex/Em 445/550 nm).

Transferrin and Folate Receptor Characterization

For the characterization of the TfR, cells were detached using Cellstripper solution (10 mins incubation at 37°C). Detached cells were then neutralized with complete media and centrifuged at 2000 RPM, 5 mins. Cell pellets were then resuspended in 3% BSA/PBS and counted. 200,000 cells were taken in each centrifuge tube. To the control, 100 µL 3% BSA/PBS was added; to the control antibody group, 100 µL of normal mouse IgG1-FITC was added (final dilution of 1:50 in cell suspension); to the transferrin antibody group 100 µL of anti-TfR IgG1-FITC was added (final dilution of 1:50 in cell suspension). These were incubated at 4°C for 30 mins following which the cell suspensions were centrifuged at 2000 RPM, 5 mins and washed with 3% BSA/PBS. Cell pellets were then resuspended in 3% BSA/PBS, kept on ice and immediately analyzed by flow cytometry (BD FACS Calibur®, Bedford, MA).

A similar procedure was used for characterization of the FR-α. 200,000 cells were incubated with the primary antibody (final dilution of 1:16), washed and incubated with the secondary

antibody (final dilution of 1:20). Antibody incubation steps were done at 4°C for 30 minutes.

Cell Association Studies

The liposomes were labeled with 1 mole % rhodamine which was included along with the other lipids prior to film-hydration and the PEG₂₀₀₀-DSPE, FA-PEG-DSPE as well as Tf-PEG-DOPE were added by post insertion as described earlier. 75,000 cells were seeded per well of a 12-well plate 24 hours prior to the experiment. Following this, the cells were treated with the formulations at a liposomal lipid concentration of 0.1 mg/mL for 4 hours. The formulations were then washed off with PBS and the cells were trypsinized, washed and maintained as a cell suspension in PBS on ice. Cells were then immediately analyzed by flow cytometry. All samples were normalized with a control cell population based on their fluorescent signal for analysis.

Particle internalization was further confirmed by a Zeiss LSM 700 confocal microscope. 10,000 cells were seeded on glass cover-slips placed in a 6-well plate 24 hours prior to the experiment. They were then treated with 0.1 mg/mL of the liposomes for 4 hours. Following this, the cells were washed with PBS and fixed with 4% paraformaldehyde and analyzed.

For the spheroid penetration studies, HeLa cell spheroids were prepared by the liquid overlay method as described previously.³⁷ Briefly, 1.5% w/v agar in serum-free media was prepared and sterilized. 50 µL of this was added to each well of a 96-well flat-bottom plate. Plates were then allowed to cool down for 45 minutes under UV. 7,500 HeLa cells were then added to each well. The plates were then centrifuged for 15 minutes at 1500 RCF at RT. The resulting spheroids were then treated with the formulations (at a liposomal lipid concentration of 0.5 mg/mL and 2mg/mL) once they were formed and compact in appearance (typically 3–5 days after seeding). The formulations were incubated with the spheroids for 4 hrs. Following this, the spheroids were harvested, washed with PBS and fixed in 4% paraformaldehyde and placed in 16-well glass-chamber slides. They were then assessed by the Zeiss LSM 700 confocal microscope. Z-stack images were then acquired starting at the apex of each spheroid at 10 µm intervals. Using the Image-J software, the difference in penetrability between each group was determined by measuring the cumulative fluorescence at stacks 5–11 (50–110 µm).

In Vitro Cytotoxicity Experiments

4000 cells were seeded in each well of a 96-well plate 24 hours prior to the experiment. Formulations were sterile filtered and incubated with the cells for 4 hours and washed off and replaced with media. The cell viability was then measured 48 hours later using the CellTiter-Blue® cell viability assay following the manufacturer's protocol. The plates were read at an excitation wavelength of 530 nm and emission of 590 nm using a BioTek Synergy HT plate reader (BioTek Instruments Inc., Winooski, VT).

In Vivo Tumor Growth Inhibition Experiments

6–8 weeks old female athymic nude mice were inoculated on the right hind-flank with 4.5×10^6 HeLa cells in 100µL of 50% v/v matrigel in serum-free RPMI media. Tumors were

allowed to develop until they were approximately 150–200 mm³ (Day 11). The animals were then randomized into six groups of four animals and injected twice a week *via* the tail vein with 100µL of formulations in PBS at an equivalent Dox dose of 4 mg/Kg. Mice were injected a total of five times with a cumulative Dox dose of 20 mg/Kg. Tumor sizes were measured using Vernier calipers and the volume was calculated using the formula,

$$\text{Volume} = (\text{Width}^2 \times \text{Length}) / 2.$$

Once the tumors in the control group reached an average size of 1000 mm³, all the animals were euthanized and the excised tumor weights as well as the volumes were measured. All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Statistical Analysis

Data was generated in triplicates and expressed as mean $\pm$ S.D. Statistical analyses were performed using a two-tailed t-test or one-way ANOVA on Graphpad Prism. Significance was determined by a P-value < 0.05 (denoted by *), P<0.01 (denoted by **) and P<0.001 (denoted by ***).

RESULTS

Characterization of Liposomes

The size distribution, polydispersity index (PDI) and zeta potential of the various liposomal formulations are summarized in Table 2. The untargeted as well as targeted liposomal formulations had an average size of ca 150 nm with a PDI of 0.13 or below showing a homogeneous size distribution. Size is an important parameter as though nanoparticles below the 400 nm size range can extravasate out of circulation into the tumor microspace, smaller sizes can internalize into the cell *via* endocytic vesicles more efficiently.^{38, 39} Analysis of the surface charge showed that the liposomes had a mean zeta potential of –21 mV to –27 mV. A zeta potential within the –20 to –30 mV range allows for increased particle stability in solution, preventing settling and flocculation of the particles as well as minimizing non-specific interactions of the liposomes with the negatively-charged cell membranes.

The Dox loading efficiency of the liposomes was determined by measuring the absorbance at 480 nm. The final Dox concentration was approximately 1.3 mg/mL for a liposomal lipid concentration of 7.5 mg/mL (encapsulation efficiency of 98%).

The release of Dox from the liposomes was then determined at pH 5.0 and pH 7.4 AT 37°C (Fig 1). It was found that at pH 5.0 more than 50% of the Dox had released by 50 hours, while at pH 7.4 only about 20% had released in 96 hours highlighting the increased retention of Dox by the liposomes at a physiological pH of 7.4.

Characterization of the Transferrin and Folic Acid Receptors on Cancer Cell Lines

The relative expression of the transferrin and folic acid receptors on the various cancer cell lines are shown in Fig 2A. It was found that compared to the HUVEC, a non-cancerous cell line, the HeLa and A2780-ADR cancer cells showed significantly higher expression of the transferrin receptor. As was expected, growing the HeLa cells in folate-deficient media (HeLa -FA) did not seem to alter the expression of the TfR.

Similarly, the FR- α was also overexpressed in the various cancer cell lines while the CCD-27Sk normal fibroblasts showed much lower expression (Fig 2B). Interestingly, growing the HeLa in folate-deficient media seemed to decrease the expression of this receptor.

Cell Association of the Liposomes in Monolayers

Cell association of the various rhodamine-labeled liposomes was carried out on HeLa cell monolayers. Initially, the effect of different ligand densities on cell association was evaluated. It was found that for optimal cell association, 0.5 mol % of the FA-PEG-DSPE and 0.05 mol % of the Tf-PEG-DOPE was required (See Suppl. Fig S1). As shown in Fig 3A, the presence of serum during the incubation step did not have a significant inhibitory effect on the ability of the liposomes to interact with the cell surface. As compared to the PL, the (Tf)L showed a significant increase in the cell association by approximately 3–4 fold. Interestingly, the (F)L did not show a significant increase in the cell association compared to PL. This can be attributed to the presence of free folic acid that is normally present in the cell media. However, the (F+Tf)L liposomes showed a 7–8 fold increase in cell association as compared to the PL, (F)L and (Tf)L. In order to negate the effects of the free folic acid, the study was repeated with HeLa cells grown in folic acid-deficient media. At these conditions (Fig 3B), the increased cell association of the (F)L over the PL was more apparent. The decrease in association of the (F)L due to competition by the 2mM free folic acid further highlights the specificity of the (F)L for the FR. Similarly at these conditions as well, the (F+Tf)L showed a dramatic 7–8 fold increase in cell association as compared to the untargeted or single-ligand targeted liposomes. The enhanced cellular internalization of the dual-targeted liposomes was further confirmed using confocal microscopy. As seen in Fig 3C, after 4 hours of incubation of formulations with HeLa cells grown in folic acid-deficient media, a significantly higher number of the rhodamine-labeled (F+Tf) L were visualized inside the cell.

Penetrability of Liposomes in a HeLa Cell Spheroid Model

Though the addition of targeting ligands allowed for better cell association and internalization, in many cases the use of targeting may cause a ‘barrier effect’ where binding of nanocarriers to the first layer of tumor cells, may prevent their further internalization into the inner layers of a solid tumor.¹⁴ This, in addition to the fact that insufficient penetration of cancer nanomedicines into the core of a solid tumor has been a long-standing issue, led us to investigate if this added affinity of the liposomes for the cell membrane would in some way inhibit their progression and or penetration in 3D-cell culture models such as spheroids. Fig 4A shows representative images from each liposome group with the cumulative fluorescence (shown in red) of the rhodamine-liposomes from 50–110 μ m layers thereby excluding the

fluorescence localized in the peripheral regions of the spheroids. Using a Z-plot, the fluorescence from each liposomal formulation was quantified as a function of spheroid depth for a 2mg/mL final liposomal lipid concentration (Fig 4B). The profile clearly indicates the increased penetration and association of the (Tf)L and the (F+Tf)L as compared to the PL and (F)L. In addition, the cumulative fluorescence from the spheroid core was quantified at two different liposomal concentrations of 0.5 mg/mL and 2 mg/mL for all the formulations (Fig 4C). This clearly indicates that the liposomes are able to efficiently penetrate into the spheroids between the cell-cell junctions by virtue of their size while at the same time demonstrating enhanced cell association in the interior layers of the spheroid. This further confirms that the addition of targeting functionalities to the liposomes do not affect their ability to penetrate further into the core of the 3D cell spheroids and associate with the cells.

In Vitro Cytotoxicity of Doxorubicin Liposomes

Next, the Dox-loaded liposomes were prepared and targeted with the appropriate ligands. The cytotoxicity of these liposomes was then evaluated on HeLa and A2780-ADR cell lines (Fig 5). Using HeLa cells which demonstrated good receptor expression of both the TfR and FR, the (F+Tf) LD exhibited enhanced cytotoxic activity over the untargeted and single-ligand-targeted groups (Fig 5A). Compared to the untargeted LD, the (F) LD also showed enhanced cytotoxicity consistent with reports in literature.^{40, 41} Thus, the addition of targeting ligands play an important role in enhancing the cytotoxic effects of liposomal Dox. The cytotoxicity of the formulations was also evaluated on Dox-resistant A2780-ADR cells showing similar response profiles (Fig 5B). As expected, the (F+Tf) LD showed significantly higher cytotoxic effects similar to that of free Dox due to the Dox-resistant nature of this cell-line. The blank liposomes as well as the blank liposomes targeted with both Tf and folic acid did not exhibit any significant cell death at the same lipid concentrations used in these experiments (data not shown). The IC₅₀ values from these studies have been summarized in Fig 5C.

Tumor Growth Inhibition in a HeLa Xenograft Model

Due to its well-characterized FR and TfR expression as well as its good response to the targeted liposomes *in vitro*, HeLa was chosen as the tumor model for the subsequent *in vivo* studies in athymic nude mice. Mice were subcutaneously inoculated with HeLa cells in 50% (v/v) matrigel to allow for good tumor formation. Since liposomes depend heavily on the EPR effect to localize into the tumor microspace, we waited until tumors were well vascularized and reached an average volume of about 150–200 mm³ before randomizing the mice into treatment groups and beginning treatments on day 11 after the inoculation. Mice were injected intravenously (IV) with the formulations at a dose of 4 mg/kg of Dox (100μL injection volume) twice a week, with a total of 5 injections administered (cumulative Dox dose of 20 mg/kg). The fold-increase in tumor volume compared to the start of treatment on day 11 was computed for each treatment group as shown in Fig 6A. Compared to the untreated group (UT), the LD, Tf (LD) and free Dox group showed significant tumor growth inhibition of 42%, 50% and 34% respectively ($P<0.05$) while the (F) LD and (F+Tf) LD showed a significantly higher tumor growth inhibition of 75% and 79% respectively ($P<0.01$). The normalized body weight % of the mice is shown in Fig 6B and no evidence of drug toxicity was observed in any of the treatment groups.

The mice were then euthanized on day 28 and the volume as well as weight of the excised tumors was measured. Representative images of the excised tumors are shown in Fig 6C. On analysis of tumor weights (Fig 6D), the (F) LD showed a significant reduction as compared to the UT ($P<0.01$), LD ($P<0.01$) and free Dox ($P<0.05$) treatment groups only, while the (F +Tf) LD showed significant reduction in tumor weights compared to the UT ($P<0.01$), LD ($P<0.01$), free Dox ($P<0.05$) as well as the (Tf) LD ($P<0.05$) treatment groups. Analysis of the excised tumor volumes (Fig 6E) yielded similar response profiles. In conclusion, both (F)LD and (F+Tf)LD were shown to be equally effective in reducing tumor burden in this selected model.

DISCUSSION

Doxorubicin is one of the most widely used chemotherapeutic agents against a broad spectrum of tumors. However, its use has been hampered by the development of cardiotoxicity in the clinic due to the action of its active metabolite.⁴ DoxilTM, the FDA-approved PEGylated liposomal form of Dox, has since emerged to overcome this issue but has not however been shown to significantly increase the rate of progression-free survival in the clinic.^{42, 43} Over the years, numerous groups have attempted to target these liposomes in order to enhance their antitumor efficacy.^{44, 45} However, it must be remembered that in spite of targeting them with ligands, they still rely predominantly on the EPR effect to reach the tumor tissues. The effect of the targeting ligand comes into play only after effective extravasation of the liposomes. A number of groups have been able to show better tumor growth inhibition by targeting Dox-liposomes with single ligands such as folic acid, CPPs, Tf etc.^{24, 40, 46} However, in some cases, these strategies have also failed to show enhancement in treatment outcomes most likely due to the heterogeneous pattern of receptor expression in tumors and the administration of a suitable dosing regimen *in vivo*. Scomparin *et al.* recently highlighted the importance of dosing frequency on the therapeutic activity of folate-targeted and untargeted liposomal Dox as well as polymeric constructs.⁴⁷ When the formulations were injected thrice at a 5mg/kg dose every-other-day, the untargeted liposomal Dox showed better tumor growth inhibition than its folate-targeted counterpart, while when three weekly injections were administered, the folate-targeted liposomes showed better tumor inhibition than the untargeted group.

As mentioned earlier, there is still a gap between *in vitro* and *in vivo* research due to the complexity of the *in vivo* setting and it is essential that we use the appropriate controls when finally evaluating the efficacy of formulations *in vivo*.³⁴ In this investigative study, four different liposomal formulations (untargeted, folate-targeted, transferrin-targeted and dual-targeted) have been compared *in vitro* and *in vivo* in order to assess their relative anti-tumor potential. These were developed in the 150 nm size range (Table 2) to allow for efficient extravasation into the tumor *via* the leaky vasculature network as well as to allow for easier internalization to provide intracellular release of Dox. Using flow cytometry, we found that the (F+Tf)L showed a remarkable 7-fold increase in cell association not only compared to the PL but also compared to the single ligand-targeted groups (Fig 3). Using HeLa cell spheroids, the ability of these nanoparticles to penetrate into the core of the spheroid was demonstrated. The transferrin-targeted as well as the dual-targeted liposomes were both able to show increased cell association with the cells in the interior of the spheroids (Fig 4). We

hypothesize that this effect was due to the increased internalization kinetics of the initial layer of liposomes, thereby allowing for the subsequent liposomal population to penetrate the spheroid interior more effectively.

Following this, the cytotoxicity of the various liposomes was then evaluated *in vitro* on HeLa as well as A2780-ADR (Fig 5) cells. Though the (Tf) LD did not show enhanced cytotoxicity when compared to its untargeted counterpart, the (F) LD showed significantly higher cytotoxic effects when compared to the untargeted LD. This is consistent with numerous reports in literature highlighting the efficiency of using folate-targeting.⁴⁸ A number of reports suggest that FR-mediated endocytosis traffics cargo directly to the sites of nucleotide synthesis such as the nucleus which may thus serve to enhance the intracellular effects of Dox by topoisomerase II inhibition.⁴¹ Compared to the (F) LD, the (F+Tf) LD group did show increased cytotoxicity as evidenced by a decrease in IC₅₀ values (Fig 5C). Compared to the cell association data, this enhancement in cytotoxic efficiency was not as drastic as we expected and can be attributed to the fact that though we do see a dramatic increase in the number of liposomes binding to the cell, their subsequent internalization by endocytosis is a fairly energy-driven process being a rate-limiting step to the delivery of Dox intracellularly.

Next, the formulations were evaluated *in vivo* using a HeLa xenograft model. After inoculation of the animals, we waited for the tumors to reach an average size of 150–200 mm³ before administering the treatments to allow for sufficient vascularization of the tumors since nanoparticles such as liposomes rely predominantly on the EPR effect to accumulate at tumor sites.⁴⁹ Interestingly, it was found that both the (F) LD as well as (F+Tf) LD showed significant tumor growth inhibition by 75% and 79% respectively compared to the UT group while when compared to LD, they showed 58% and 64% more inhibition respectively, successfully highlighting the advantages of combining targeting moieties (Fig 6).

Overall, though the dual-targeted liposomes showed a significant increase in cell association and cytotoxicity compared to their untargeted as well as single-ligand targeted counterparts *in vitro*, in the *in vivo* setting, it was found that both the dual-targeted as well as folic-acid targeted liposomes were able to equally inhibit tumor growth. Though this study highlights the advantages of using targeting moieties to enhance the therapeutic response of nanomedicines in the treatment of solid tumors *in vivo*, it also shows that simultaneously targeting formulations with multiple ligands has the potential to increase in some cases, their complexity considerably *in vivo* without significant therapeutic advantages although in other cases it can be beneficial.^{27–31} It is thus evident that although *in vitro* experimental designs allow for the easier evaluation of formulations as well as at higher throughput, it is imperative that these formulations be investigated *in vivo* as well with their appropriate controls before being given any clinical potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PEG	Polyethylene glycol(methoxy-PEG ₂₀₀₀ -DSPE)
Tf	transferrin
TfR	transferrin receptor
FR	folate receptor
Dox	doxorubicin
CPP	cell penetrating peptides
MPS	mononuclear phagocytic system
EPR	enhanced permeability and retention
ePC	eggphosphatidylcholine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
CHEMS	cholesteryl hemisuccinate
DCC	dicyclohexylcarbodiimide
MWCO	molecular weight cut-off
FBS	fetal bovine serum
TLC	thin-layer chromatography
PBS	phosphate buffered saline
HPLC	high performance liquid chromatography
BSA	bovine serum albumin
PL	untargeted PEGylated rhodamine-labeled liposomes
(F)L	folic acid-targeted rhodamine-labeled liposomes
(Tf)L	transferrin-targeted rhodamine-labeled liposomes
(F+Tf)L	folic acid- and transferrin-targeted rhodamine-labeled liposomes
LD	doxorubicin liposomes
(F) LD	folic acid-targeted doxorubicin liposomes
(Tf) LD	transferrin- targeted doxorubicin liposomes

(F+Tf) LD folic acid- and transferrin-targeted doxorubicin liposomes

UT untreated

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Figure 1.
Cumulative release of Dox from liposomes at pH 5.0 and pH 7.4 at 37°C.
Data are represented as mean $\pm$ S.D. (n = 3)

Figure 2.
Characterization of (A) transferrin and (B) folate receptor expression.
Data are represented as mean $\pm$ S.D. (n =3)

Figure 3.

(A) Cell association of liposomes on HeLa cells in media with folic acid and (B) without free folic acid as determined by flow cytometry (n=3) followed by (C) their internalization as determined by confocal microscopy on monolayers (scale bar = 15µm) Data are represented as mean $\pm$ S.D.

Figure 4. Penetration of rhodamine-labeled liposomes into HeLa cell spheroids
 (A) Representative images of slices 50–110 μm in depth (scale bar = 50 μm). (B) Profile of fluorescence with increasing depth into the spheroid (2 mg/mL liposomes). (C) The cumulative fluorescence of slices (50–110 μm) at 2 different liposomal concentrations as quantified using Fiji data analysis software. Data are represented as mean $\pm$ S.D. (n = 4)

Figure 5.

In vitro cytotoxicity of the various liposomal formulations on (A) HeLa cells (B) A2780-ADR and (C) summary of IC₅₀ values. Data are represented as mean ± S.D. (n=3)

Figure 6. Evaluation of formulations in a HeLa xenograft model

(A) Tumor growth inhibition. (B) Normalized body weight (%). (C) Representative images of excised tumors. (D) Excised tumor weights. (E) Excised tumor volumes. Data are represented as mean $\pm$ S.D. (n = 4)

Table 1

Composition of Liposomes

Lipid	Mole Percentage %
ePC	66%
Cholesterol	24%
Dioleoyl-sn-glycero-phosphoethanolamine (DOPE)	5%
Cholesteryl hemisuccinate(CHEMS)	5%
PEG ₂₀₀₀ -DSPE	3%
Tf-PEG ₃₄₀₀ -PE	0.05%
FA-PEG ₃₄₀₀ -PE	0.5%

Table 2

Characterization of Liposomes

Formulation	Size ^a (nm)	PDI ^a	Zeta Potential ^b (mV)
Untargeted PEGylated Liposome (PL)	149 +/- 42	0.1	-21 +/- 1
Folate-Liposome (F) L	152 +/- 39	0.1	-23 +/- 1
Transferrin Liposome (Tf) L	164 +/- 43	0.14	-27 +/- 1
Dual-targeted Liposome (F+Tf) L	165 +/- 33	0.13	-21 +/- 1.5