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RESEARCH ARTICLE

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Antennapedia-derived positively-charged peptide faces multiple problems upon their usage as targeting ligand for liposomal doxorubicin

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Abstract

The positively-charged peptide antp derived from Antennapedia transcription protein is demonstrated to mediate the liposome translocation across the cell membrane. In the current investigation, we prepared a stable liposomal doxorubicin (Dox) formulation and targeted it with the antp peptide from 0 to 200 ligand/liposome. These antp-containing liposomes were investigated in terms of physical stability on storage in the refrigerator and upon incubation in blood. Also, other features like cell binding, uptake, biodistribution, and treatment efficiency were evaluated in C26 colon carcinoma BALB/c mice. The Antp in liposomes resulted in enhanced particle growth with the development of the enormously large liposomes from 2000 to 6000 nm. Upon incubation in blood, these large liposomes were removed. The antp also enhanced the cell binding affinity and cell uptake rate of the liposomes and resulted in the restriction of the cancer cell proliferation, but it failed to improve the chemotherapeutic property of the Dox-liposome. The i.v. injection of antp-liposomes (15 mg Dox/kg) caused severe body weight loss and early death incidence due to probably increased toxicity. The antp targeting offered no advantage to the Dox-liposome in the delivery of Dox to the tumor, and failed to enhance the treatment efficiency of the liposomes.

KEYWORDS
Antennapedia, cell delivery, liposomal doxorubicin, size distribution, tissue distribution, tumor treatment

1 | INTRODUCTION

Peptide transduction domains (PTD) are short basic (positively charged) sequences present in some viral and cellular proteins that mediate their transfer across the cell membrane.[1] These PTDs are artificially used in a variety of drug delivery systems, including inorganic nanoparticles, polymeric nanoparticles, micelles, and liposomes to enhance the rate of drug delivery.[2,3] Concerning liposomes, different PTDs have been used for targeting purposes (Dox).[4–6] Although it is revealed that the post-insertion of PTD into liposomes enhances the drug delivery to the tumor cells, the PTD-insertion brings some costs and benefits in tumor therapy. Some PTD-liposomes were found to face some pharmacokinetic and therapeutic limitations when the liposomes were injected into circulation.[4,7] In our previous studies, targeting liposomal Dox with TAT-trans-activator of transcription (TAT) peptide sequence was found to reduce the half-life of the drug in the blood circulation and change the tissue distribution profile of the drug in a way that reduces the therapeutic efficacy of the

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liposome.[4,8] Targeting liposomal Dox with PNC27 hybrid peptide – composed of the HDM2 binding 12–26 amino acid domain of p53 protein and the positively-charged sequence of Antennapedia (antp) transcription proteins- also resulted in similar outcomes as with TAT.[5] Nonetheless, PNC27-liposomal Dox enhanced the delivery of Dox to the tumor and improved the therapeutic efficiency to some extent. Therefore, the therapeutic response might be tuned based on the type of PTD used.

PNC27 improved the anti-tumor activity of the Dox-liposome but showed by itself no anti-cancerous effect. The control Dox-free liposome post-inserted with PNC27 showed no anti-cancer cytotoxicity nor anti-tumor activity in C26 colon carcinoma cells that ruled out the anti-cancerous function of the HDM2 binding domain. The PNC27 insertion into Dox-liposome promoted the delivery of Dox to the cells, though; denoting the possible effectiveness of the antp part in increasing the delivery of Dox to the tumor.

In the current report, a truncated version of the PNC27 peptide that lacks the HDM2 binding domain is used in Dox-liposomes to examine various physical, anti-tumor, and bio-distributive features of these liposomes. The idea for this study lies in the fact that the antp peptide itself might improve the delivery of the liposomal Dox to the tumor tissues.

2 | MATERIALS AND METHODS

Antp peptide, Ac-CGGGKKWKMRRNQFWVKVQRG was purchased from China peptides Co. (Shanghai, China). Hydrogenated soya phosphatidylcholine (HSPC) and 1,2-distearoyl-phosphatidyl ethanolamine-methyl-polyethyleneglycol conjugate (mPEG2000-DSPE) were purchased from Lipoid (Ludwigshafen, Germany). Maleimide-PEG2000 distearyl-phosphatidyl ethanolamine (Mal-PEG2000-DSPE) was obtained from Avanti Polar Lipids (Alabaster, AL). DiD (D307) lipophilic tracer was purchased from Invitrogen (Thermo Fisher, CA). Cholesterol (Chol), doxorubicin hydrochloride (Dox), cation exchanger resin (Dowex 50WX4 hydrogen form, 200–400 mesh), Dulbecco's Modified Eagle's medium (DMEM) and RPMI 1640 culture medium were purchased from Sigma-Aldrich (St. Louis, MO); MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from Promega (Madison, WI); and isopropanol was bought from Merck (Darmstadt, Germany). All other solvents and reagents were used as chemical grade. Pharmaceutical grade liposomal doxorubicin (Caelyx®) was purchased from Behestan Darou Co. (Tehran, Iran).

2.1 | Antp-PEG-lipid conjugation

The antp peptide was attached covalently to the Mal-PEG_{2000}-DSPE via a thioether bond. To this end, the peptide and the lipid were first dissolved in dimethyl sulfoxide (DMSO) and chloroform, respectively. Then, they were added to a glass flask at the molar ratio of 1.2/1 and incubated there under nitrogen atmosphere and continuous mild agitation,[9] The peptide–lipid reaction was monitored during the incubation using silica thin layer chromatography (TLC). The developing solvent of the TLC was chloroform/methanol/water (90/10/2 v/v) and the spots were developed under iodine vapor on the TLC paper. After 48 h incubation, the solvent was evaporated under a warmed nitrogen stream followed by overnight freeze-drying. The white powdery product was then re-suspended in deionized water (2 mg/mL).

Furthermore, the coupling reaction was determined by RP-HPLC (Lachrom Elite HPLC system, Shimadzu, Japan) on Kromasil 100–5 C18 column (4.6 mm × 250 mm, 5 microns, Kromasil, Sweden). The buffers were water +0.1% trifluoroacetic acid (TFA) and acetonitrile +0.1% TFA. The chromatograms were collected at 220 nm using a solvent gradient of 20%–50% acetonitrile +0.1% TFA in 12 min with a flow rate of 1 mL/min.

Finally, the antp-PEG-DSPE conjugate suspension was subjected to excessive dialysis against NaCl (1 M) and distilled water[9] and the efficacy of the reaction was also determined using Tricine-SDS-PAGE and silver staining on a three-sectional gel consisted of the stacking (4%), spacing (10%), and separating (16%/6 M urea) gel parts.[10]

2.2 | Liposomes

Liposomes were prepared by the thin lipid film hydration and extrusion. To prepare Dox-liposome, the liposome was loaded with Dox using the ammonium sulfate gradient method.[11] In this process, an appropriate amount of the lipids, previously dissolved in chloroform as a stock solution, was added to a round-bottom flask according to Table. 1. Then, the chloroform solvent was removed with a vacuum-equipped rotary evaporator followed by freeze-drying. The resultant thin lipid film was then hydrated with pre-warmed ammonium sulfate (250 mM) to a final total lipid concentration of 25 mM, and the suspension was passed through polycarbonate nanopore filters of 100 nm and 80 nm pore size (Avastin, Canada). Afterward, the liposome was dialyzed against HEPES buffered sucrose (HBS; 10 mM HEPES, 300 mM sucrose, pH 7.0) in dialysis cassettes (Pierce, Rockford, IL) with 12–14 kDa molecular weight cut off (MWCO). Finally, the liposomes were incubated with Dox hydrochloride at the ratio of 1 mg Dox/7 μmol total phospholipid at 65°C for 90 min to achieve the liposomal Dox.

Fluorescently labeled liposomes were also prepared using the same procedure as for the liposomal Dox. Briefly, DiD (D307) lipophilic tracer was added to a glass flask at a 0.2 mol% ratio along with other lipids. Likewise, the solvent was removed with the successive evaporation and freeze-drying technique, the lipid film was hydrated with HBS, and the resulting suspension was extruded through the polycarbonate membranes.

Subsequently, the previously prepared antp-lipid was inserted into the Dox- and DiD-liposomes at various ratios. To this end, antp-PEG-DSPE micelle suspension was added to these liposomes at appropriate volumetric ratios to achieve theoretically 0, 25, 50, 100, 200 antp/liposomes in a tube. The tube was then incubated in a hot bath at 60°C for 3 h, and the liposome-micelle suspension was mixed occasionally.[12] Next, the tube's content was passed through an

TABLE 1 Physical properties of the liposomes

Nomenclature	Liposome composition (molar ratio)	Z-average (nm)	PDI[a]	Peak intensity	Z-potential	Liposomal DOX (mg/ml)
Liposomal Dox	HSPC, Chol, $mPEG_{2000}$-DSPE (56.2: 38.3: 5.3)	100 ± 35[b]	0.130	100%	10.0 ± 4.9	2.1 ± 0.1
Liposomal Dox/25-antp	HSPC, Chol, $mPEG_{2000}$-DSPE, antp (56.2: 38.3: 5.3, 0.03)	108 ± 36	0.206	99%	11.1 ± 4.4	2.3 ± 0.2
Liposomal Dox/50-antp	HSPC, Chol, $mPEG_{2000}$-DSPE, antp (56.2: 38.3: 5.3, 0.06)	117 ± 38	0.220	96%	11.2 ± 4.4	1.7 ± 0.2
Liposomal Dox/100-antp	HSPC, Chol, $mPEG_{2000}$-DSPE, antp (56.2: 38.3: 5.3, 0.12)	127 ± 53[c]	0.302[a]	87%	11.3 ± 4.6	1.5 ± 0.1[c]
Liposomal Dox/200-antp	HSPC, Chol, $mPEG_{2000}$-DSPE, antp (56.2: 38.3: 5.3, 0.24)	141 ± 55	0.357[a]	83%	9.5 ± 5.5	1.2 ± 0.2[c]

[a]Polydispersity index.
[b]The values are presented as mean ± width of the primary peak.
[c]Shows significant difference as compared to liposomal Dox ($P < 0.05$).

equilibrated sepharose CL-4 B column to remove the probably unmerged antp-lipid-micelles.

2.3 | Physicochemical characterization of the liposome

The liposome size distribution and polydispersity index (PDI) were measured by dynamic light scattering (Zetasizer Nano-ZS; Malvern, UK). The liposomal phosphorous content was measured as per the method described by Bartlett[13] with some modifications and used as the total phospholipid concentration of the liposomal formulation. For this, an appropriate amount of the liposomes (~80 nmol of total phospholipids) and 0.65 mM phosphorus standard solution (from 30 to 150 nmole, Sigma-Aldrich, St. Louis, MO) were added to 6 mL glass tubes containing 0.4 mL H_2SO_4 (10 N). The tubes were covered by glass marbles and heated at 180°C for 60 min in a dry heater block (Techne, UK). Subsequently, 0.1 mL of hydrogen peroxide (10% v/v) was added and the glass heated again for further 10 min. Having been left at room temperature for 10 min, 4.5 mL of ammonium molybdate reagent (1.89 mM in 0.25 mM H_2SO_4) and 0.5 mL of 0.1 g/mL ascorbic acid were added to the tubes containing the colorless liquid. After shaking, the tubes were put into a water bath at 100°C until the tubes color turned dark blue. Finally, the absorbance of the tubes' content was measured at 800 nm by spectrophotometry (UV-1900 UV–VIS Spectrophotometer, Shimadzu, Japan) and the phosphorous content of the liposomes was calculated against the phosphorous standard curve.

The liposomal Dox concentration was measured by spectrofluorimetry (ex: 470 nm/em: 590 nm, PerkinElmer LS45) against DOX standard curve in an acidified isopropanol.[14] For this, the liposome samples (25 μL) were added to vials containing 40 mg of the Dowex resin in 1 mL cold phosphate buffered saline (PBS; pH 7.4) and shaken for 1 min. Immediately, 0.1 mL of the supernatants was transferred into new vials containing 2 mL of the acidified isopropanol (90% isopropanol/0.075 M HCL). The vials were then heated at 60°C for 30 min and the Dox was measured. Similarly, the DiD content of liposomes was measured by the spectrofluorimeter (ex: 600 nm/em: 665 nm) in the acidified isopropanol against DiD standard curve.

2.4 | In vitro Dox release

Dox release from the liposome was monitored within an hour in vitro in a medium. To this end, the liposome (0.1 mL), Dowex resin (120 mg), and PBS/FCS (3 mL, 50/50 v/v) were added to a vial and incubated at 37°C. The vials were given a mild shake every 5 min during the 1 h incubation period, and at several intervals, samples were withdrawn. For negative (0% release) and positive control (100% release), samples of the liposome were added to cold NaCl (600 mM) and hot deionized water +0.1% Triton X-100, respectively; along with the Dowex resin. For these tubes, the samples were withdrawn after a quick shake by hand from the negative control tubes and at 20 min of the incubation period in the hot water bath (90°C) from the positive control tubes. All mentioned samples were dissolved in the acidified isopropanol, and their absorbance was measured with the spectrofluorimeter against a Dox standard curve as described in the previous section.

Subsequently, the mean absorbance values of the test, negative and positive samples were used in the following equation (Equation (1)) to achieve the percentage of Dox release from the liposomes at different time points.

$$Dox\ release\ (\%) = \frac{(Dox\ conc._{Samples} - Dox\ conc._{positive}) \times 100}{(Dox\ conc._{Negative} - Dox\ conc._{Positive})} \quad (1)$$

2.5 | Liposome size distribution study

The particle size growth of liposomes was monitored in time in the above-mentioned media and also in human blood for some of our

studied liposomes. These were Dox-liposome, Dox-liposome/200-antp, DiD-liposome, and DiD-liposome/200-antp. These liposomes (0.1 mL) were added to separate vials containing 3 mL of the PBS/FCS (50/50 v/v) and the human blood that was collected from the lab staff volunteers into EDTA-treated BD Vacutainer collection tubes (Becton Dickinson, USA). The vials were incubated at 37°C in a cell incubator under continuous agitation, from which the samples were taken at 0, 6, 24 h post-incubation. For the liposomes in the PBS/FCS medium, the samples (0.2 mL) were washed (× 2) with excessive cold normal saline in Amicon™ Ultra-4 centrifugal filter devices (Amicon® Ultra 100 kDa device, Sigma-Aldrich, St. Louis, MO) under centrifugation (1000g for 10 min). For the liposomes in the blood, the blood (0.5 mL) was first transferred to a fresh vial and centrifuged at 1000g for 10 min, then the serum (0.2 mL) was washed (×3) with excessive cold normal saline in the centrifugal filter-equipped tubes. Finally, the liposome size distribution was measured by the dynamic light scattering.

2.6 | Cells

Three cell lines were studied in this report with the following cell culture specifications. These were B16F0 melanoma and NIH/3T3 cell lines (Sigma-Aldrich, St. Louis, MO), which were cultured in DMEM, and C26 colon carcinoma cell (Eppelheim, Germany), which was cultured in RPMI 1640 medium. Both media were supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere, and their viability was assessed by the trypan blue dye exclusion method before the experiment.[15]

2.7 | Cell-association studies

The cell-liposome studies were conducted with the C26 cell line using a flow-cytometric method.

Briefly, Dox-liposomes (20 μg/well), DOX (20 μg/well), and Antp-peptide (10 μg/well) were incubated with 1×10^6 viable cells - in a cell suspension at 4°C for the cell-binding affinity study and a 12-well culture plate at 37°C for the cellular uptake study. The liposomes were incubated without and with the Antp peptide at a competitive dose for 3 h with the cells, which were harvested at the end of the incubation period. Finally, the Dox-associated fluorescent intensity of the cells was measured on the FL2 channel of the flow-cytometer with the detector in logarithmic mode (FL2-H).

2.8 | Cytotoxicity

The cell toxicity of the f-Dox and Dox-liposomes was determined in the three cell lines using MTT assay.[16] Briefly, B16F0 melanoma, NIH/3T3, and C26 colon carcinoma cells (1×10^4 cells/well) were incubated in separate 96-well plates for 24 h in a cell culture incubator. Subsequently, the plates' medium was replaced with a fresh one (0.2 mL FCS-free culture medium) supplemented with serial dilutions of the liposomes and Dox. At 3 and 6 h of the incubation period, the plate's medium was again replaced this time with the culture medium. The plates were then incubated for 24 h in this medium, and then for another 3 h after being supplemented with the MTT reagent (20 μL/well, 5 mg/mL in PBS). Next, the medium was removed completely and replaced with DMSO (0.2 mL/well) to dissolve the purple-colored cells in the wells. After complete dissolution of the wells' contents, their absorbance values were recorded at 550 nm by a Multiskan plus plate reader (labsystems). Finally, half-maximal inhibitory (IC50) concentration of Dox for each test reagent was calculated with the help of a four-parameter logistic curve (Equation (2)) in the "log dose vs. response" panel.

$$Y = \frac{Bottom + (Top - Bottom)}{\left(1 \times 10^{((LogIC50 - X) \times Hillslope)}\right)} \quad (2)$$

where Y and X are the absorbance values and the relevant Log Dox dose, respectively, and Top and Bottom are the maximum and minimum values that reached plateaus.

All animal experiments were performed in compliance with the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences guidelines. Four to six-week-old female BALB/c mice were received C26 cells (3×10^5 cells/mouse) in the right flank subcutaneously (SC). Upon the emergence of a palpable tumor, the mice were allocated randomly to different groups and injected with the liposomes (15 mg Dox/kg) via tail vein. The control group received dextrose 5% solution as a placebo. Subsequently, mice were followed in terms of body weight change and tumor size or any sign of severe suffering and death incidence regularly till the end of the experiment by an investigator blind to the groups' treatment. The tumor volume (mm^3) was measured with a digital caliber using three orthogonal diameters (a × b × c × 0.5 mm) of the tumor. These mice were followed till they met the criteria for euthanasia – including body weight loss (20%>) and tumor volume (1000 mm^3 >)[17,18] – and till groups reach the median survival time (when three out of five case died).

2.9 | Biodistribution

The Dox decay rate in serum and Dox tissue distribution were examined for the biodistribution study in the tumor-bearing BALB/c mice (three per group) following the injection of the liposomes. Twelve days after tumor inoculation when the C26 tumors were approximately 5 mm in diameter, an i.v. injection of the liposomes was given to the mice, whose blood was collected from the retro-orbital plexus at 3, 6, and 12 h afterward. The final blood sample was achieved with a heart puncture at 24 h when the animals were sacrificed. Subsequently, the tumor, one of the kidneys, spleen, heart, lung, and a piece (~150 mg) of liver and muscle were removed, weighed, and

transferred to 2 mL polypropylene vials (Biospec, OK) containing 1 mL acidified isopropanol and zirconia beads. These tissues were completely homogenized with a Mini-Bead beater-1 (Biospec, OK). Next, the supernatant (0.4 mL) was withdrawn after centrifugation at 21,000g for 5 min, diluted with an appropriate volume of acidified isopropanol, and stored overnight at 4°C. The next day, the Dox concentration of the samples were measured with the spectrofluorimeter against the Dox calibration curve prepared in tissue and serum extracts of the control mice.[17,18]

2.10 | Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). Two-tailed statistical analysis was conducted at a significance level of 0.05. Moreover, one-way ANOVA and the post-test Newman–Keuls multiple comparison were used. The nonlinear regression model used to calculate IC50 values (Equation (3)) was compared with the pooled model according to the Equation (3). Time-to-end point (TTE), which is the time (expressed in day) at which tumor size reach 1000 mm^3, was calculated using log-linear regression analysis of the log tumor size (*Y*-axis) versus the day post-tumoring (*X*-axis), when the measurement was made. Log-rank test was used for comparison in TTE values between treatment groups in the event-free survival graph. When a tumor volume passed 1000 mm^3, the final tumor size recorded for the animal was used to calculate the mean size at the subsequent time points, according to Schluep et al.[19]

3 | RESULTS AND DISCUSSION

3.1 | The validation of Antp-PEG-lipid conjugation

It was a pre-requisite step to validate the linkage of antp-peptide to the Mal-PEG_{2000}-DSPE lipid before their usage in the liposome. Hence, the coupling reaction was validated in the current study via three methods of TLC, RP-HPLC, and SDS-PAGE (Figure 1). The coupling reaction was primarily determined by the TLC (Figure 1(a)). In the lipid lane (L), the lipid spot (b) moved with the mobile phase across the TLC, while the peptide spot (P) remained at the start line in the peptide lane (P). As with the lipid spot, the spot in the reaction lane moved along the TLC that indicated the attachment of the peptide to the lipid. The efficiency of the linkage was confirmed with the RP-HPLC (Figure 1(b)). There found two peaks for the reaction tube at 4.4 min and 7.6 min retention times (Figure 1(bC)). Upon the injection of the free antp peptide to the column, a sharp peak emerged at exactly 4.4 min retention time, where the smaller peak of the reaction tube had appeared (Figure 1(bD)). This indicated that the first peak at 4.4 min Rt is probably related to the remnants of the peptide that remained uncoupled. This peak was spiked with the addition of a small amount of the free antp peptide to the reaction tube, confirming that the peak at 4.4 min Rt is indeed for the free-peptide (Figure 1(bE)). The other peak at 7.6 min Rt was much bigger than the first one. Integrating the area under the curve (AUC) showed that the second peak is 4.2 times bigger than the first one. Contrary to the first peak, the second peak showed no change in its AUC with the addition of the free-peptide to the reaction tube, and the free PEG2000-DSPE showed no peak on the chromatogram (data not shown). Taken all these pieces of evidence together, the second peak was related to the coupled antp-PEG2000-DSPE. Moreover, the linkage efficiency of 88.5% was estimated for the peptide and the lipid according to the relative AUC of the mentioned peaks. This linkage efficiency matched with the initial relative mole ratio of the peptide and the lipid, where 20% more peptide (in mole ratio) had been added to the reaction tube.

Furthermore, the linkage reaction was also confirmed by SDS-PAGE (Figure 1(c)). The reaction (R) and peptide (P) bands were found at about 4.4 kDa and 2.0 kDa in comparison with the protein molecular markers. This 0.6 kDa increase in weight from the R band to the P band signified the attachment of the peptide to the lipid.

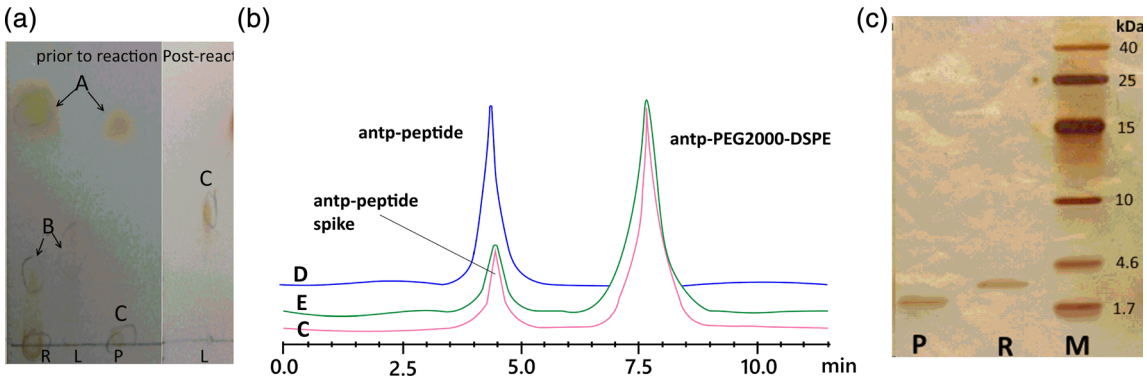

FIGURE 1 Evaluation of the Antp-peptide conjugation to DSPE-mPEG2000-mal with TLC (a), RP-HPLC (b), and SDS-PAE (c). A, B and C indicate the DMSO, DSPE-mPEG2000-mal, and the antp-peptide-related spots, respectively. D and E are the chromatograms obtained from the antp-peptide stock solution, the reaction tube, respectively. F is the chromatogram obtained from the reaction tube that had already mixed with a given amount of the free peptide before the injection into the column. P, R, and M signifies the lanes for the peptide, reaction tube product, and molecular markers

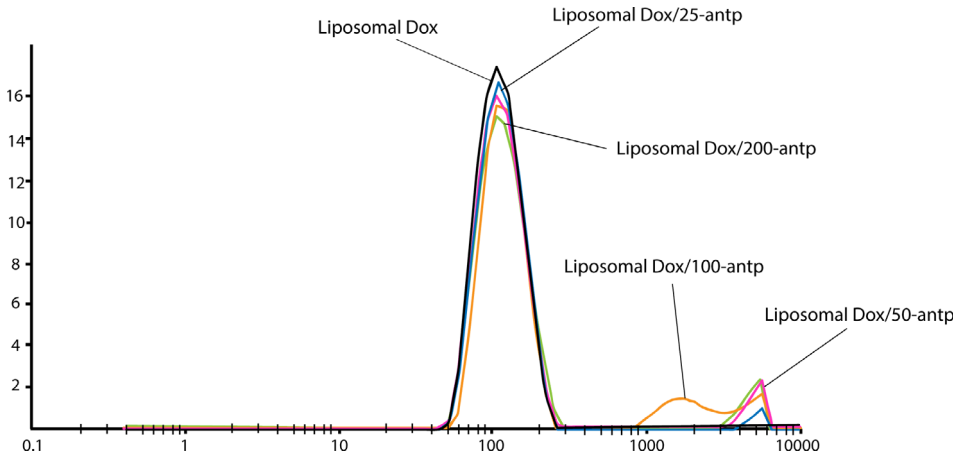

FIGURE 2 Particle-size distribution profile of antp-liposomal Dox formulations and the antp-free liposomal Dox (the black curve). *Y*- and *X*-axes show the differential intensity of the particles (in percentage term) and their associated sizes in (in nanometer scale and logarithmic mode)

3.2 | Physical properties of the liposomes

Upon liposome preparation, almost all liposomes targeted with the antp peptide displayed a population of enormously large particles in their particulate size distribution (Figure 2). The population of the enormously large particles seemed to be proportional to the amount of antp-lipid already used in the liposomal formulation. Approximately 16% of the particles were in the range of 1000–10,000 nm in the liposomal Dox/100-antp formulation. Liposomal Dox/200-antp displayed 12.7% of an average 3100 nm particles with a broad size distribution from 1000 to 6000 nm. About 5% of the particles were in the size range of 5000–10,000 nm in the liposomal Dox/50-antp. This population of large particles dropped to 2.1% in the liposomal Dox/25-antp, and no large particles were found in the antp-free liposomal Dox formulation.

Targeting liposomes with the antp peptide, therefore, resulted in the development of large liposomes. It is worth mentioning that it was the peptide–lipid, not the Dox that had led to the particle heterogeneity as the control Dox-free liposome/200-antp showed also a population of extra-large particles in their particle size distribution. As with the liposomal Dox/200-antp, 3.5% of the particles had an average size of 5000 nm with a broad size distribution from 3000 to 6000 nm in this liposomal formulation.

Other physicochemical parameters, however, remained stable in the series of the liposomes. The liposome surface charge (*z*-potential) remained constant for all the liposomes (at about −10 mV), so did the ratio of the Dox to the lipids in the liposomes (at about 12.6 mole %). This indicated that targeting the liposomes with the peptide does not affect the surface charge, nor the membrane permeability of the liposomes.

When the antp-liposomes were pumped through the Minisart® syringe filters (cellulose acetate membrane, 0.45 m pore size, 16,555 K, Sigma-Aldrich, St. Louis, MO), however, the level of Dox decreased considerably to 6.4 mole % in the liposomal Dox/200-antp. This indicated that a considerable portion of the Dox was encapsulated in the enlarged particles. These results were in contrast with those of our previous studies, in which mono-modal particle size distribution (∼ 100 nm with PDI < 0.2) was found in PNC27-liposomes and TAT-liposomes.[4,5]

3.3 | Liposome size distribution following incubation in blood

The particle size distribution of the liposomes changed considerably after incubation in the blood (Figure 3). The curve related to the

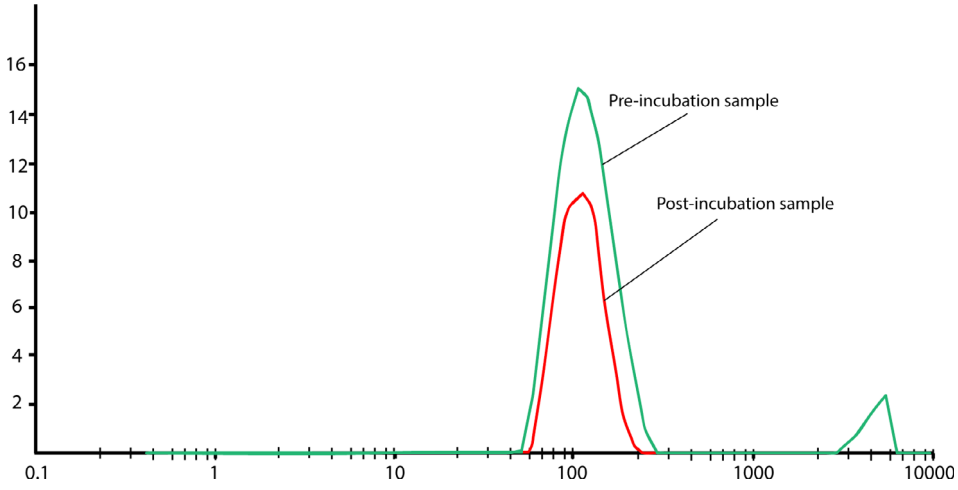

FIGURE 3 The particle size distribution profiles of the liposomal Dox/antp-200 before and after 3 h-incubation period in human blood

population of the extra-large liposomes disappeared after the incubation, and those related to the population of the small liposomes (with the average size of 120 nm) narrowed down. In other words, all large (300 nm) liposomes were removed from the serum within the incubation period. This indicated that the larger liposomes are more liable for rapid removal by the blood cells than the smaller liposomes.[20,21]

3.4 | Dox release

In addition to the particle size growth, it seemed that the peptide–lipid brings instability in the liposomal membrane. This could be seen in the enhanced leakage of Dox from the liposomes (Figure 4). The presence of the antp-lipid increased the release of Dox from the liposomes, especially in the liposomes with more than 100 peptides/liposome (Figure 4). A similar finding is reported with other cell-penetrating peptides as the ligand for the liposomes. In our previous study, we reported that the incorporation of TAT peptide into liposomal formulation enhances the leakage of Dox from the liposomes as well.

3.5 | Liposome-cell experiment

Figure 5 shows the binding affinity and cellular uptake of the liposomes following incubation at 4°C and 37°C.

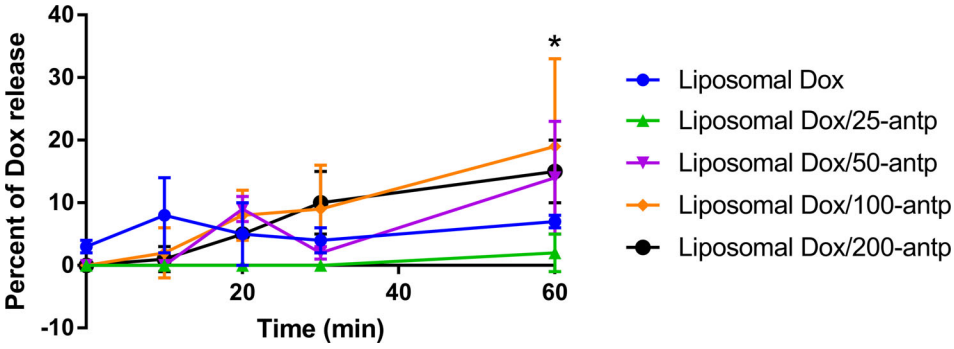

FIGURE 4 Percent of Dox release from the liposomes in PBS/FCS (50/50 v/v) at 37°C. * indicates significant difference between liposomal Dox/100-antp, liposomal Dox/200-antp and the antp-free liposomal Dox formulations. Data are shown as mean ± standard deviation of three independent samples ($P < 0.05$)

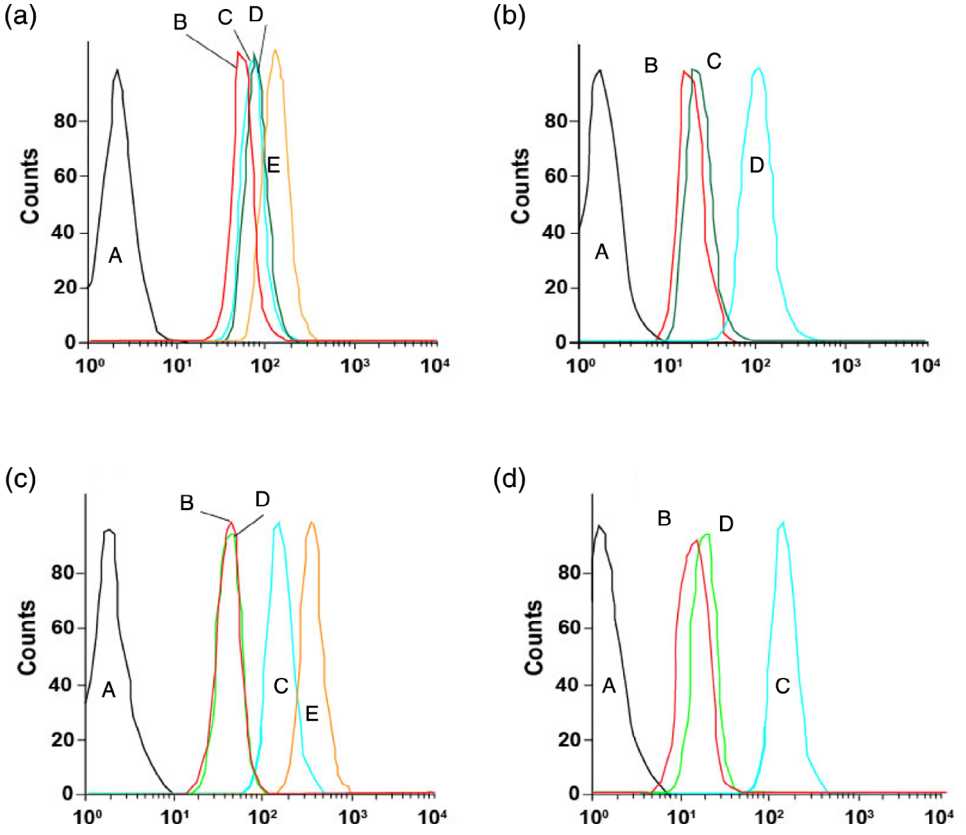

FIGURE 5 Histograms related to the cell binding and uptake of the Dox-loaded and the DiD-loaded liposomes at two different temperatures. (a) and (b) show the Dox-associated fluorescent intensity of the C26 colon carcinoma cells, at 4 and 37°C, respectively. In parallel, (c) and (d) display the DiD-related fluorescent intensity of these cells at these temperatures. Considering the histograms, A is for the control cells left untreated, B and C are for those treated with the antp-free and the antp-targeted liposomes, and D is the histogram of the cells treated with the antp-targeted liposomes and high amount of the antp peptide. E is for the unliposomal Dox

TABLE 2 Half-maximal inhibitory concentration (IC50) of the liposomes and Dox solution

Treatment	C26 cells	B16F0 cells	NIH 3 T3 cells
Liposomal Dox	14.5 ± 3.5[a]	32.6 ± 12.0	67.7 ± 21.3
Liposomal Dox/25-antp	12.4 ± 2.2	35.7 ± 14.4	65.3 ± 23.2
Liposomal Dox/50-antp	6.5 ± 1.3	28.6 ± 8.9	55.6 ± 14.7
Liposomal Dox/100-antp	3.3 ± 0.6	18.3 ± 5.4	46.9 ± 8.9[b]
Liposomal Dox/200-antp	3.8 ± 0.8[b]	13.6 ± 7.1[b]	36.5 ± 6.8[b]
f-Dox	0.3 ± 0.1[b]	2.7 ± 1.3[b]	8.6 ± 1.8[b]
Dox-free liposome/200-antp	35.5 ± 8.9	96.7 ± 34.5[b]	187.0 ± 43.3[b]

[a]Data are shown ± standard deviation ($n = 8$).
[b]Shows significant difference as compared to liposomal Dox ($P < 0.05$).

Both the liposomal Dox and liposomal Dox/200-antp were found to have equal cell binding affinity. These liposomes transferred a similar amount of Dox to the C26 cells at 4°C (Figure 5(aB) vs. (C)). The cell-binding affinity of the liposomal Dox/200-antp showed no change in the presence of the excessive amount of the free peptide also (Figure 5(aD)).

The incubation of the Dox-liposomes showed different results at 37°C (Figure 5(b)). The cellular uptake of Dox increased significantly in the liposomal Dox/200-antp compared to the naïve antp-free liposomes (Figure 5(bB) vs. (C)). Such an increase in the level of cellular Dox was associated with the targeting role of the peptide present in the liposomal formulation. Such an effect was masked by incubating the cells with the antp-liposomes at the presence of a high concentration of the free peptide. There was the same amount of Dox in the cells treated with the liposomal Dox/200-antp and the antp-free liposomes in the presence of the competitive dose of the free antp peptide (Figure 5(bD) vs. (B)).

The above experiment was repeated with the DiD-model liposomes to achieve a better view of the cell-liposome interaction. Since the Dox could also be transferred indirectly from the liposome to the cell after release from the liposome, DiD-model liposome with the hydrophobic fluorescent tracer in their membrane was prepared and then incubated with the cells (Figure 5(c)). Like the histogram of cells with Dox, the DiD signal distribution in the cells showed similar binding affinity to the cells for both the antp-targeted and the antp-free liposomes at 4°C (Figure 5(cB) vs. (C)). The cellular uptake of DiD-liposome was increased with the antp-targeted liposome, and it decreased back to the same level at the presence of the competitive dose of the free peptide (B vs. D). Therefore, an improvement as such could be attributed to the specific interaction of the peptide with the cell ligands,[1] since DiD-liposome/200-antp transferred the same amount of DiD to the cells as DiD-liposome did in the presence of the excessive dose of the free-antp peptide.

Targeting the liposomes with the antp-peptide also increased the level of Dox and DiD cellular uptake. At 37°C, the cell could actively take the liposomes from the medium via endocytosis.[22] This allowed the more pronounced transition of liposomal Dox and DiD-liposomes to the cells, which seemed to be mediated by the antp-peptide targeting. Both the level of Dox and DiD cell uptake increased significantly in the liposomes targeted with the antp-peptide. As with the cell binding affinity assay, the addition of excess amounts of the free-antp peptide prevented such increased cellular uptake of the liposomes, which indicated the cell-specificity of the antp-mediated liposomal delivery.[1] It is reported that the membrane translocation of the PTD is mediated by the cell surface-expressed glycosaminoglycans, in particular heparin sulfate glycosaminoglycans, and in this regard, TAT and Antp peptide, might have differential binding affinity to these negatively-charged extra-cellular glycosaminoglycan moieties.[1] This might result in the cell type specificity of the liposomes and target-oriented Dox delivery.

It was determined that the addition of 200 antp peptide per liposome (0.25% molar ratio) could significantly enhance the cytotoxicity of the liposomal Dox in all three cell lines (Table 2). The addition of antp peptide to liposomal formulation of Dox decreased the half-maximal inhibitory concentration (IC_{50}) of Dox from 4.18 ± 1.23 for the liposomal Dox/200-antp to 2.40 ± 1.47 for the liposomal Dox in C26 cells 3 h post-incubation. The liposomal Dox/200-antp also decreased the IC_{50} significantly as compared to the antp-free liposomal Dox in B16F0 melanoma cells and NIH 3T3 normal cells. Surprisingly, it was found that Dox-free antp-liposome could limit the cell proliferation, indicating that the antp-liposome could exert cytotoxic effect on these cells, regardless of the cell type.

3.6 | In vivo studies

The single injection of the antp-liposomes led to some serious outcomes and changes in the biodistribution profile of the liposomal Dox.

Figure 6 illustrates the chemotherapy-related outcomes in the C26 tumor-bearing mice. The injection of the antp-liposomes had some serious repercussions without further therapeutic gain as compared to the naïve Dox-liposome at dose 15 mg Dox/kg. Some subjects died within the first week in all groups treated with the antp-liposomes (Figure 6(a)), while no death was seen in the antp-free liposome-treated and placebo groups within this period. Among the liposomes, the antp-free liposomal Dox was the best treatment in terms of survival rate. The antp-containing and antp-free liposomes both led to a significant body weight loss in mice as compared to the placebo within the first weak after the injection (Figure 6(b)), but the antp-free liposome-treated mice gained more weight afterward. Considering tumor growth,

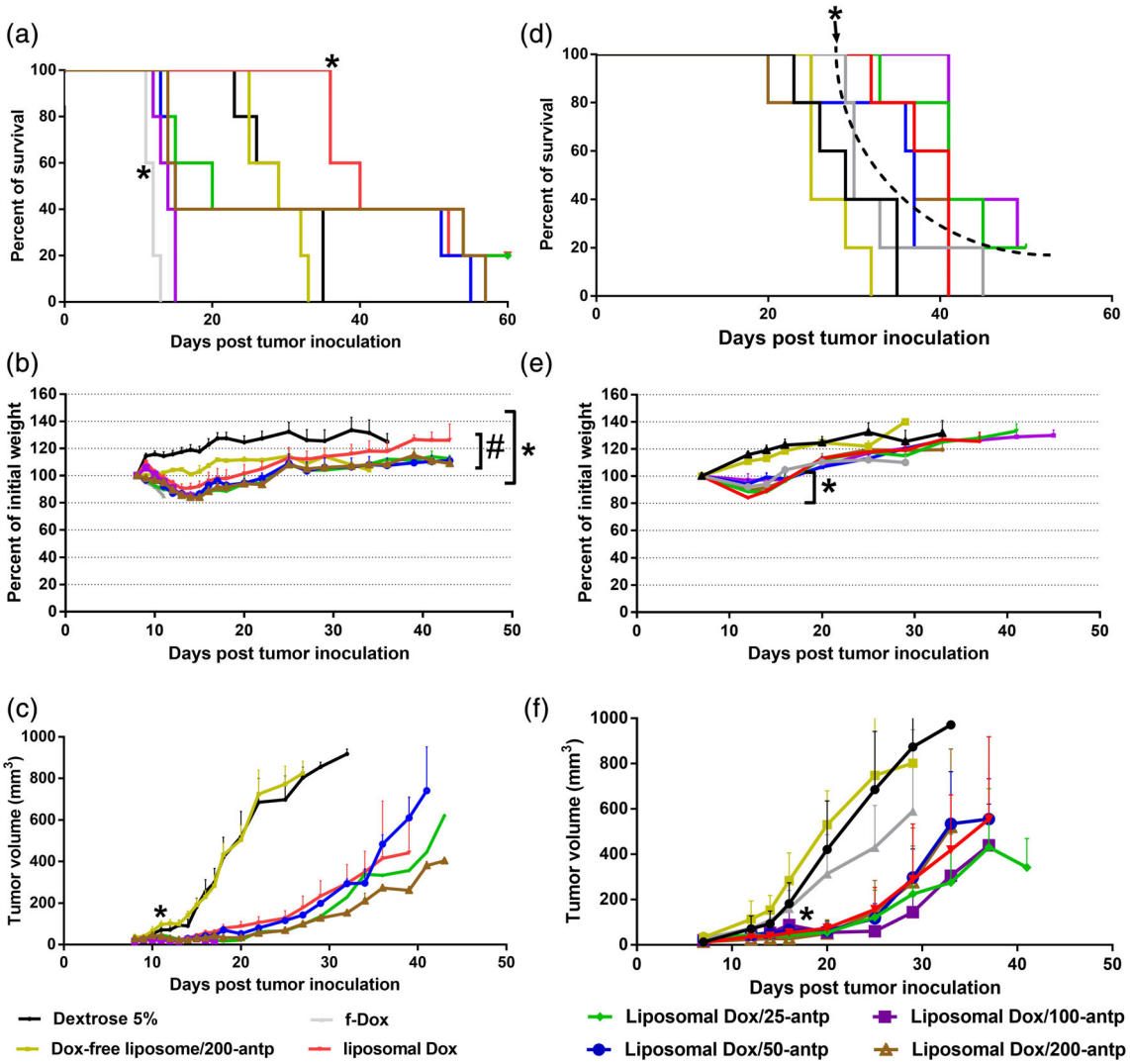

FIGURE 6 The event-free survival, body weight change and tumor size growth charts of the female BALB/c mice injected with different liposomes. (a), (b), and (c) are the charts for the mice injected i.v. with 15 mg Dox/kg body weight, and (d), (e), and (f) are for those treated at 10 mg Dox/kg dose. * and # indicate significant difference as compared to the liposomal Dox and the control group injected with dextrose 5% solution. The cut-line curve with the star on top in the (d) graph shows the borderline between the groups with a significant difference. Data are shown as mean ± standard deviation of five subjects at $P < 0.05$

the antp-containing liposomal Dox restricted tumor growth to the same extent as the antp-free liposomes.

Given the fact that some mice in the antp-liposomes' groups died in the first week, the experiment was repeated at the lower dose of Dox (i.e., at 10 mg/kg, Figure 6(d)–(f)). With the decrease in the injection dose from 15 to 10 mg Dox/kg, the acquired median survival time remained unchanged for the antp-free liposomal Dox at 40th day post- tumor inoculation (Figure 6(d)). Considering other groups, there was no early death incidence within the first week in any group, and the injection of the liposomes at 10 mg Dox/kg still failed to show any further improvement in the survival median time than that of liposomal Dox. At this dosage, both antp-containing and antp-free liposomal Dox resulted in the same trend of weight body change (Figure 6(e)) and also the same trend of tumor growth till the end of the experiment (Figure 6(f)). So, targeting the Dox-liposome with the Antp peptide offered no further therapeutic benefit, nor more tumor growth restriction as compared to the corresponding antp-free Dox-liposome.

The control unliposomal Dox and the Dox-free liposomes provided no therapeutic benefit as compared to the placebo (Dextrose 5%) group. The non-liposomal Dox at 15 and 10 mg/kg was too toxic to be tolerated and led to the death incidence within the first day of the injection and a significant loss of weight in the mice. Similarly, the Dox-free liposome showed no improvement in the survival median time.

Figure 7 shows the serum level of Dox in time and the tissue distribution at 24 h post-injection of the liposomes. In general, there was a similar distribution profile for all the liposomes, except for some differences between the biodistribution of antp-liposomes and the antp-free liposome. Three hours after the injection of the liposomes at

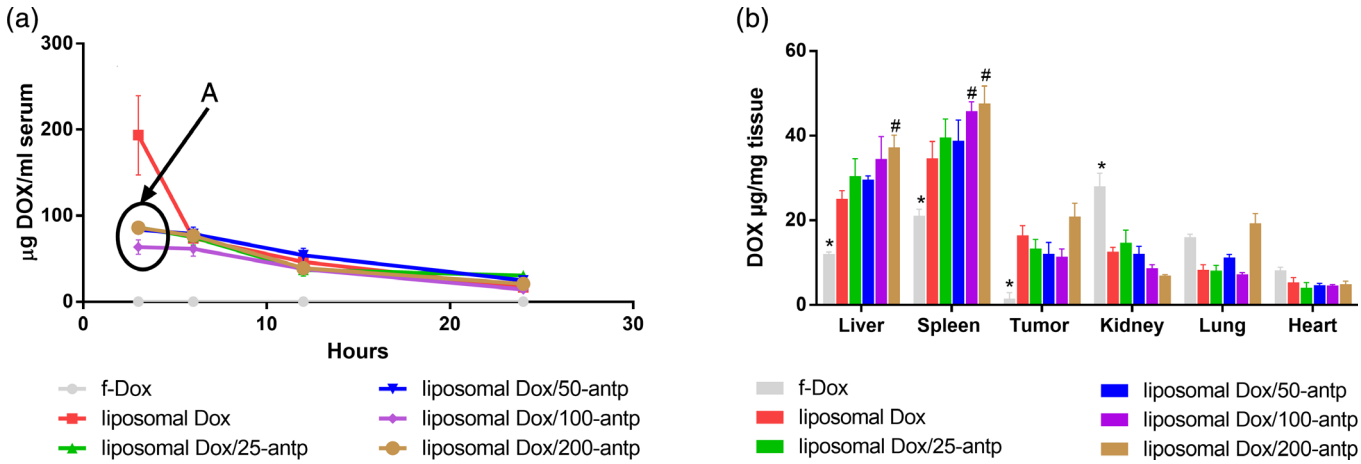

FIGURE 7 The concentration of Dox in serum and different tissues following i.v. injection of the liposomes to C26-colon carcinoma-bearing BALB/c mice. (a) shows the serum concentration of the Dox within 24 h incubation of the liposomes, and (b) displays the related tissue concentration of Dox at the end when the animals were sacrificed. For comparison, the serum and tissue concentration of the Dox is also assessed in a group injected with the non-liposomal, free Dox solution (f-Dox). There was a noticeable difference in the serum concentration of Dox at 3 h between the liposomal Dox and all antp-containing liposomal Dox (depicted in the circle, A). * and # indicate a significant difference with the antp-targeted Dox-liposomes and liposomal Dox. Data are shown as mean ± standard deviation of three subjects ($P < 0.05$)

15 mg Dox/kg body weight, the serum concentration of Dox in the antp-liposome injected mice was half the concentration found in the serum of mice injected with the antp-free liposome (Figure 7(a), 100 vs. 200 μg Dox/ml serum). The serum Dox concentration showed a similar reduction trend in all liposomal Dox formulations afterward. This was in agreement with the in vitro experiment finding, where the incubation of the antp-liposomes in blood resulted in the fast removal of the enormously large liposomes and their associated Dox. The measurement of the tissues' concentration of Dox at 24 h post-injection revealed considerably higher amounts of Dox in the spleen and liver tissues of the mice injected with the liposomal Dox/200-antp than the mice treated with the liposomal Dox (Figure 7(b)). However, targeting with antp peptide failed to enhance Dox delivery to the tumor tissues as compared to the liposomal Dox.

Although the liposome modification with antp-peptide failed to improve Dox delivery to the tumor tissue, the encapsulation of Dox in liposome indeed improved the tumor concentration of Dox. Our previous study showed that TAT-liposomal Dox failed to increase Dox delivery to the tumor tissues and cells too[23,24] but, generally, the biodistribution profile of Dox was improved in the form of the liposome. The encapsulation of Dox in liposomes provides prolonged serum retention, limited glomerular filtration rate, and sustained tumor delivery as mentioned in the literature.[4]

Liposomal modification with the antp peptide led to the increased accumulation of Dox in the spleen and liver tissues of the mice. Such an increase could be attributed to the population of the enormously large liposomes (>100 nm) present in these formulations.[25,26] It is shown that the large particles (350 nm) can infiltrate the fenestrated vasculature system of the liver and spleen tissues easily, while most tumors have small perforations in their vessels that limit traversing particles larger than 100 nm in size.[27] In addition, larger nanoparticles are removed from serum more rapidly than smaller nanoparticles via the reticuloendothelial system.[28] Given the mentioned changes in the biodistribution profile, the enormously large liposomes might be the main culprit for the poor pharmacokinetic and increased toxicity of Dox. The rapid removal of the large Dox-liposomes by blood cells and their increased accumulation in the vital organs like the liver, spleen, and kidney might be associated with the severe toxicity of the antp-liposomes. It also might be the main reason for not improving the delivery of Dox to tumor.

4 | CONCLUSION

Although antp-peptide incorporation into a liposomal formulation enhanced the cellular delivery of the drug, it also led to some serious problems as evidenced in our study. The antp-peptide incorporation was accompanied by enhanced Dox release from liposomes and particle size growth in the Dox liposomal formulation. A considerable amount of Dox was encapsulated in the large liposomes, which was found to be removed rapidly by blood cells and in the blood circulation. In addition, the addition of antp- peptide to the liposomal Dox resulted in the enhanced Dox accumulation in the liver and spleen tissues, but not tumors. These liposomes caused severe symptoms, reduced the bodyweight of the animals and the maximum tolerated dose of the liposomal Dox. Taken together, the antp-liposome faced serious repercussions which make their use inappropriate for the enhanced cell-targeting purpose of the liposomes.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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