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Research paper

Targeted delivery of RGD-modified liposomes encapsulating both combretastatin A-4 and doxorubicin for tumor therapy: In vitro and in vivo studies

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ABSTRACT

Arg–Gly–Asp (RGD) modified doxorubicin-loaded liposomes could improve anticancer effect, and vascular disrupting agents (VDAs) could induce a rapid and selective shutdown of the blood vessels of tumors. We propose that RGD-modified liposomes for co-encapsulation and sequential release of vascular disrupting agent combretastatin A-4 (CA-4) and cytotoxic agent doxorubicin (Dox) could enhance tumor inhibition responses. In this study, we encapsulated Dox and CA-4 in RGD-modified liposomes. The release rate of Dox was proved to be much slower than that of CA-4 in vitro. Flow cytometry and laser confocal scanning microscopy clearly showed that RGD-modification promoted intracellular uptake of liposomal drugs by B16/B16F10 melanoma tumor cells and human umbilical vein endothelial cells (HUVECs). Cytotoxicity assay showed that the IC_{50} of RGD-modified liposomes was lower than that of the corresponding unmodified liposomes. Therapeutic benefits were examined on B16F10 melanoma tumors subcutaneously growing in C57BL/6 mice. In vivo study demonstrated that RGD-modified liposomes exhibited the most pronounced tumor regression effect when both CA-4 and Dox were co-encapsulated. These results suggest that the targeted drug delivery system for co-encapsulation of vascular disrupting agents and anticancer agents may be a promising strategy for cancer treatment.

1. Introduction

The superiority of combination therapy for cancer treatment has been well known for several decades. Antivascular therapy and cytotoxic therapy are complementary: circulating tumor cells, very early metastases and the dividing rim of mature tumors are sensitive to tumor-targeted therapies, whereas angiogenic metastases and more mature tumors are most sensitive to antivascular therapies [1]. However, the combination of these two drugs, typically when given separately [2], faced several problems: (i) In a long-term treatment which is needed to suppress tumor growth, anti-angiogenesis agent impairs blood flow inside the tumor microenvironment, precluding the access of cytotoxic agent and accumulation of next dosages. (ii) Activation of hypoxic response leads to increased metastases and resistance to chemotherapy [3]. An effective solution is to choose an agent which causes reversible vascular disruption and deliver the cytotoxic drug into tumor site before vascular collapse caused by antivascular agent. For instance, Sengupta et al. developed a vehicle called "nanocell" [4], a multidrug-loaded delivery system which releases the drugs sequentially. Focal drug release within tumor results in improved therapeutic index with reduced toxicity. This study may represent a successful attempt of sequential release strategy for combination chemotherapy.

Liposomal delivery has become a well-established method in cancer treatment [5,6]. While sterically stabilized liposomes (SSL) can passively accumulate into tumor tissue due to the effect of enhanced permeability and retention (EPR), the subsequent intracellular uptake of the entrapped anticancer drugs by the tumor cells remains an inefficient step [7]. Based on the fact that the integrin (RGD-dependent receptor) is overexpressed in the melanoma tumor cells and tumor endothelial cells [1], we recently validated that RGD-modified micelles significantly facilitated the intracellular delivery of the encapsulated agents into human umbilical vein endothelial cells (HUVEC) and melanoma B16 cells via integrin-mediated endocytosis [8].

Aiming at establishing an "integrative" drug delivery system for sequential release of CA-4 and Dox, we developed RGD-modified liposomes loaded with both CA-4 and Dox. One potential advantage of our liposomes compared to "nanocell" was the surface

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modification with RGD peptide, which may enhance the intracellular drug uptake by tumor endothelial cells and melanoma tumor cells. Another benefit would come from a more complete release of doxorubicin from liposomes than from doxorubicin-PLGA oligomers, which is not bioactive until decomposed [4]. To test its targeting effect, the specific cell internalization of liposomes was investigated on B16F10 melanoma tumor cells and human umbilical vein endothelial cells (HUVECs) in vitro. In addition, the antitumor activity was evaluated via i.v. injection in C57BL/6 mice bearing B16F10 melanoma.

2. Materials and methods

2.1. Materials

Arginine–glycine–aspartic acid, RGD (Zhongkeyaguang Biotechnology Co., Ltd., Beijing, China), egg phosphatidylcholine (EPC) (Lipoid GmbH, Co., Germany), Cholesterol (Chol) and Sephadex G50 from Pharmacia Biotech (Piscataway, NJ, USA). Doxorubicin hydrochloride (Dox) was obtained from Haizheng Pharmaceutical Co. (Zhejiang Province, China). Combretastatin A-4 was supplied by Fude Chemical Co., Ltd. (Shanghai, China). (Methoxypolyethelene-glycol (Mw = 2000)–distearylphosphatidylethanolamine (DSPE-PEG) and DSPE-PEG-NHS (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*n*-[poly(ethyleneglycol)]-hydroxy succinamide, PEG Mw = 2000) were purchased from NOF Co. (Tokyo, Japan). Endothelial cell growth supplement (ECGS), collagenase type I and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cell culture media RPMI 1640, M199, penicillin–streptomycin, fetal bovine serum, L-glutamine and HEPES buffer were from GIBCO, Invitrogen Corp. (USA). Hoechst 33258 was purchased from Molecular Probes Inc. (USA). Histiocyte stationary liquid was purchased from Saichi Biotechnology Co. (Beijing, China). Dialysis Tubing (Mw cutoff 3500, 14,000) was purchased from Jingkehongda Biotechnology Co., Ltd. (Beijing, China). Methanol and acetonitrile (Merck Chemical Co.) were of HPLC grade. All other solvents and reagents were of analytical grade and used as received.

The murine melanoma cells B16F10 were obtained from the Basic Medical Cell Center, Chinese Academy of Medical Science (Beijing, China). Male C57BL/6 mice (6–8 weeks old), ranging from 18 to 22 g, were provided by Vital River Laboratory Animal Center (Beijing, China). All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care.

2.2. Synthesis of RGD–PEG-DSPE

The activated DSPE-PEG-NHS was used to conjugate RGD to DSPE-PEG. In brief, the RGD and DSPE-PEG-NHS (RGD/DSPE-PEG-NHS: 1/2) was dissolved in *N*,*N*-Dimethylformamide (DMF) [9], stirring continuously at room temperature for 36 h. Following reacting for 10 h, triethylamine (DSPE-PEG-NHS/TEA: 1/2, mol/mol) was added to the reaction mixture [10]. The reaction endpoint was traced by HPLC method until the RGD nearly disappeared. Finally, the reaction mixture was dialyzed extensively (dialysis bag MW cutoff 3500) against distilled water for 48 h to remove all impurities and then lyophilized.

2.3. Liposomes preparation

Accurately weighed amounts of lipids and drug (EPC/Chol/DSPE-PEG/CA-4: 25/1.28/6.24/2, mg/mg) were dissolved in chloroform in a round-bottom flask, dried into a transparent thin film on a rotary evaporator under vacuum at 37 °C. The molar ratio of EPC/cholesterol/DSPE-PEG was 65/30/5 [11]. The resulting lipid film was then hydrated with 2 mL physiological saline at 50 °C by vigorously vortex and sonicated in a water bath at 50 °C for 20 min. The targeted liposomes were prepared in an identical procedure replacing the pure DSPE-PEG with the mixture of DSPE-PEG-RGD and DSPE-PEG synthesized as described above. Free CA-4 was then removed by G50 gel-filtration.

For the preparation of doxorubicin-loaded liposomes, the physiological saline was replaced by the same volume of 50 mM $(NH_4)_2SO_4$ solution. The CA-4-loaded liposomes were passed through a Sephadex G50 (pharmacia Biotech, NJ, USA) gel-filtration column pre-equilibrated in physiological saline to exchange the external phase. Dox was then remote-loaded via an ammonium sulfate gradient method. In brief, CA-4 liposomes were pre-heated in 37 °C water bath, and proper amount of 2 mg/mL Dox solution was added to incubate with the liposomes at 37 °C for 15 min, stirring gently to load the Dox into the liposome inner phase. Free Dox was then removed by gel-filtration. Liposomes were stored at 4 °C and used within 24 h of preparation.

2.4. Characterization of drug loaded liposomes

Dox and CA-4 concentrations in the liposomal samples were calculated from peak area of HPLC at 233 nm and 295 nm, respectively, following dissolution in 0.1% Triton X-100. The hydrodynamic diameters, polydispersity index (PDI) and Zeta potential of CA-4-loaded liposomes (L[C]), CA-4 and Dox-loaded liposomes (L[CD]) and RGD-modified CA-4 and Dox-loaded liposomes (RGD-L[CD]) were measured using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malven, UK). The analysis was performed with 10 mW He–Ne laser (633 nm) at scattering angle of 90° at 25 °C. The liposome was diluted with PBS (PH 7.4) before measurement. The results were determined three times for each sample.

For encapsulation efficiency measurement, a 100-μL liposome suspension was passed through a Sephadex G50 gel-filtration column, and the collected liposomes were dissolved in 900 μL methane, then 100 μL of the above mixture was diluted to 10 mL with the mobile phase. The contents of CA-4 and Dox were measured by HPLC method. The encapsulation efficiency was calculated as follows: Encapsulation efficiency% = (peak area after gel-filtration × 1000/peak area before gel-filtration) × 100%.

To determine the release kinetics of CA-4 and Dox from liposomes, L[CD] or RGD-L[CD] (1 mL) was placed in a dialysis tubing (molecular weight cutoff size 14,000) and dialyzed against 250 mL physiological saline at 37 °C for 48 h with gentle shaking. At designated time points, 100 μL aliquots were withdrawn from the incubation medium and replaced with equal volume of the fresh medium. At the end of 48 h, the dialysis bags were cut open and 2.0 mL 10% Triton X-100 was mixed thoroughly with the release medium. The concentrations of Dox and CA-4 were then measured by HPLC to calculate the maximum drug release amount.

2.5. Cell culture

Integrin-overexpressing B16 and B16F10 melanoma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (streptomycin 100 μg/mL, penicillin 100 U/mL) at 37 °C in humidified air with 5% CO_2. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured with the method we previously described [8].

2.6. Cellular uptake of liposomes by flow cytometry analysis

An aliquot of 1.0 mL of melanoma B16, B16F10 or HUVEC cells suspension (1×10^5 cells/well for B16 and B16F10, 5×10^4 cells/well for HUVEC) was seeded in a six-well tissue culture plate

(Corning, NY, USA) and incubated for 24 h at 37 °C until cells were grown nearly confluent. Then, the medium was replaced with 1.0 mL Dox-loaded liposomes (L[D]) or RGD-modified Dox-loaded liposomes (RGD-L[D]), diluted with FBS free culture medium, with final Dox concentration of 40 μg/mL. The plates were incubated at 37 °C. At various time points (20 min, 1 h and 3 h), the medium was removed and cell monolayer was suspended by brief treatment with trypsin and then washed three times with cold PBS. Then the cell samples were examined by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA). The intracellular Dox was excited with an argon laser (488 nm), and fluorescence was detected at 560 nm. Files were collected of 10,000 gated events and analyzed with the FCS Express software.

2.7. Intracellular distribution of liposomes by confocal microscopy analysis

B16 cells, B16F10 cells and HUVECs were seeded on sterile 35-mm glass-base dishes. Cell density is 5×10^5 cells/well for melanoma cells and 2.5×10^5 cells/well for HUVECs. The cells were incubated for 24 h to 50% confluence and then incubated with L[D] or RGD-L[D] for 3 h as described above. The cells were then washed three times with cold PBS, fixed by histiocyte stationary liquid (Saichi Biotech, Beijing, China) at room temperature for 10 min, followed by cell nuclei staining with Hoechst 33258 for 5 min before washed three times with PBS for confocal microscopy analysis. Leica SP2 confocal microscope (Heidelberg, Germany) was used to detect the intracellular uptake of Dox (excitation/emission wavelength: 480 nm/540 nm) and investigate their cellular distribution. Fluorescent images of cells were analyzed using LSCM fluorescence intensity quantitative software.

2.8. In vitro cytotoxicity assay

Cytotoxicities of various liposomes were tested on B16F10 cells. Briefly, each well of 96-well plates was seed with 5000 cells and incubated for 48 h. The cells were then exposed to serial concentrations of L[C], L[D], L[CD] and RGD-L[CD] diluted in culture medium. After cultured for 36 h at 37 °C, the cell monolayer was washed with PBS. Then, 20 μL of MTT solution (5 mg/mL) was added to each well, followed by incubating for another 4 h at 37 °C. Finally, the medium was replaced by 150 μL of DMSO, and the absorbance was read on Sunrise Absorbance Microplate Reader (TECAN, Austria) at wavelength of 540 nm. The experiment was carried out in triplicate. The data reported represent the means of triplicate measurement.

2.9. In vivo therapeutic efficacy

The tumor regression effect of liposomes was investigated in male C57BL/6 mice (6–8 weeks old, 18–22 g) inoculated melanoma B16F10 cells (1×10^6) subcutaneously in the right flank of the mice. After the tumor volume increased to 50 mm^3 a week later, the mice were randomly sorted into five groups (6 mice/group), and each group was treated by tail vein injection every 48 h with one of the following: physiological saline (control); CA-4 and Dox-loaded liposomes (L[CD]); RGD-modified CA-4-loaded liposomes (RGD-L[C]); RGD-modified Dox-loaded liposomes (RGD-L[D]); RGD-modified CA-4 and Dox-loaded liposomes (RGD-L[CD]). The dose of CA-4 and Dox was 25 mg/kg and 0.8 mg/kg, respectively. Five injections were performed in the treatment process. Tumor volumes and mouse body weights were measured every alternate day during treatment. Measurement of tumor size was performed with a caliper in two dimensions, and individual tumor volumes (V) were calculated by the formula: $V = [\text{length} \times (\text{width})^2]/2$. The mice were executed two days after the last injection. Then the mice body weights and tumor weights were measured. The net body weights (the body weights subtract the tumor weights) were calculated as an indication of in vivo toxicity.

2.10. Statistical analysis

All data were reported as means ± standard deviation (SD). For statistical analysis between two groups, Student's t-test for independent means was used. A value of $P < 0.05$ was considered as statistically significant, and a P-value less than 0.01 was considered as highly significant. The differences between the overall therapeutic effects of different treatments on tumor growth were analyzed by one-way analysis of variance (ANOVA) with LSD Multiple Comparison Test. Statistical analysis was performed using the SPSS software (SPSS Inc, Chicago, IL).

3. Results

3.1. Synthesis of RGD–PEG-DSPE

Preparation of DSPE-PEG-RGD was performed as described previously [12]. Chromatographic analysis of the reaction mixture at different time points (Fig. 1) showed that the RGD was consumed almost completely under the conditions of reaction. Around 50% of input DSPE-PEG-NHS in reaction system was conjugated with RGD peptide. The resulting product was then used for preparing liposomes without further purification.

3.2. Characterization of drug loaded liposomes

The particle sizes of various liposomes were around 90 nm (PDI < 0.3) (Table 1), and all types of liposome have similar particle sizes and polydispersity index. It was reported that a liposome diameter of about 100 nm is likely to be a optimal size, not only for the more effective extravasation of liposomes, but also for their longer retention in tumor tissue [13]. The encapsulation efficiency of CA-4 in various liposomes was around 70–80% and the encapsulation efficiency of Dox was above 95%. The drug loading efficiencies were similar for liposomes with and without RGD modifications (Table 1).

Fig. 2 shows that CA-4 and Dox were released from L[CD] at rates comparable to those observed for RGD-L[CD]; however, CA-4 was released much faster than Dox in both targeting and non-tar-

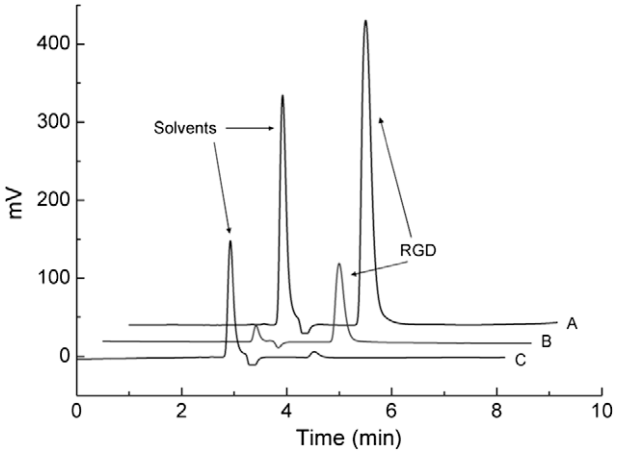

Fig. 1. High-performance liquid chromatography analysis of RGD conjugation to DSPE-PEG-NHS at different time points: (A) 0 h, (B) 20 h, and (C) 36 h.

Table 1
Characteristics of the prepared liposomes ($n = 3$).

Liposomes	Average liposome size (nm)	Polydispersity (PDI)	Zeta potential (mV)	Entrapment efficiency (%)	
				CA-4	DOX
L[C]	91.15 ± 3.42	0.27 ± 0.01	−0.88 ± 0.46	76.8 ± 4.8	–
L[CD]	91.20 ± 1.84	0.26 ± 0.01	−7.08 ± 0.68	73.3 ± 1.1	98.1 ± 0.3
RGD-L[CD]	90.54 ± 0.34	0.27 ± 0.01	−1.01 ± 0.65	73.9 ± 1.5	97.4 ± 0.6

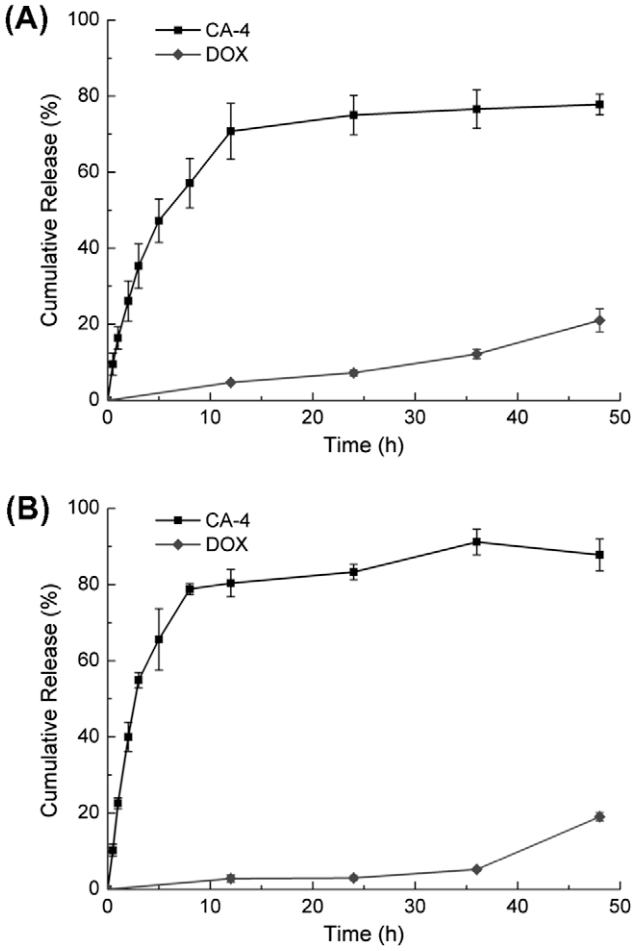

Fig. 2. In vitro release of CA-4 and Dox from L[CD] (A) and RGD-L[CD] (B) in physiological saline at 37 °C ($n = 3$).

geting liposomes. The CA-4 accumulative release rate was close to 80% after 24 h, but only 20% of Dox was released after 48 h.

3.3. Cellular uptake of liposomes

Cellular uptake of liposomal Dox was enhanced by RGD modification. Fig. 3A showed the fluorescence intensity in B16F10 melanoma tumor cells at different times following treatment with L[D] and RGD-L[D]. The amount of L[D] uptake was increased with incubation time, which were 20 min, 1 h and 3 h. In addition, RGD-L[D] showed greater uptake by the cells compared to L[D] at each time point, which demonstrated that RGD coupling lead to increased cellular uptake of liposomes. The two liposomes were also incubated with B16 cells (Fig. 3B) and HUVECs (Fig. 3C), respectively, and similar results were obtained. Fig. 4 shows the flow cytometry analysis of fluorescence intensity for each cell line at 1 h following incubation with liposomes. These data consolidated the result of Fig. 3.

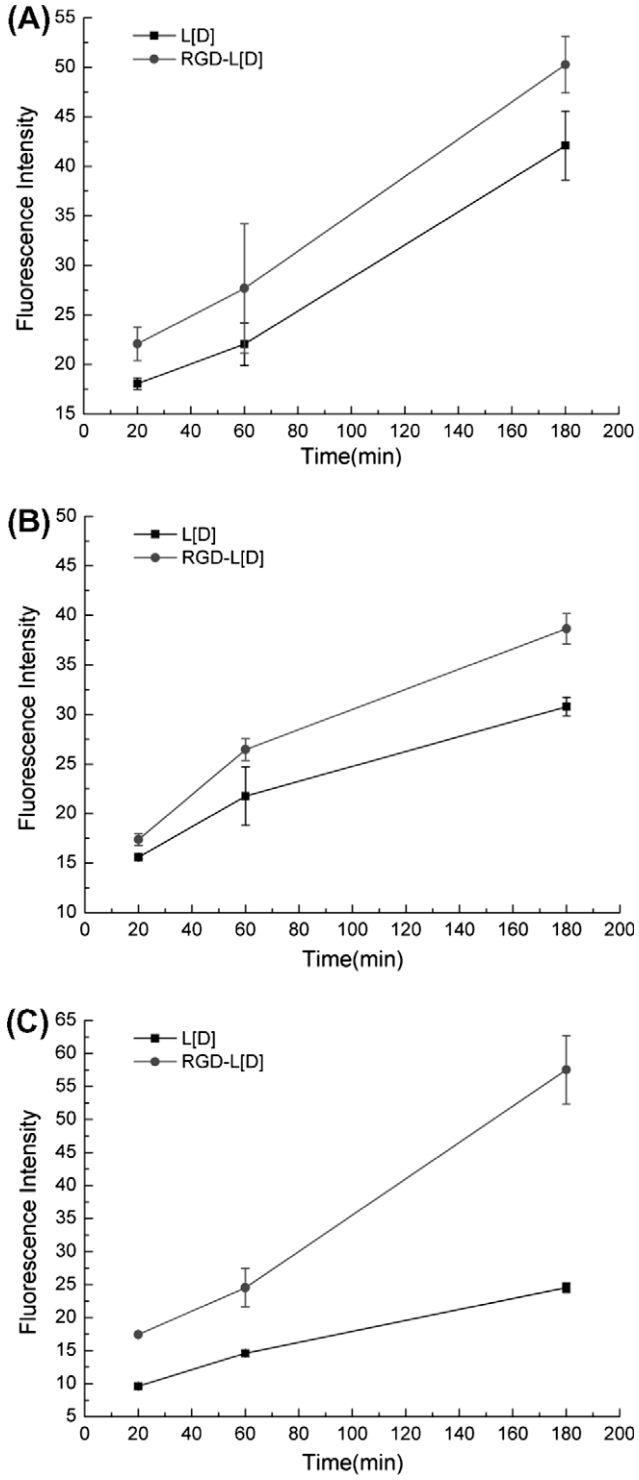

Fig. 3. Intracellular Dox fluorescence intensity in B16F10 (A), B16 (B) or HUVEC (C) cells treated with L[D] (■) or RGD-L[D] (●). Cells (1×10^6 cells/well) were incubated with various liposomal Dox at a final concentration of 40 μg Dox/ml diluted in culture medium at 37 °C. The fluorescence intensity was detected with flow cytometry at different time points following treatment ($n = 3$).

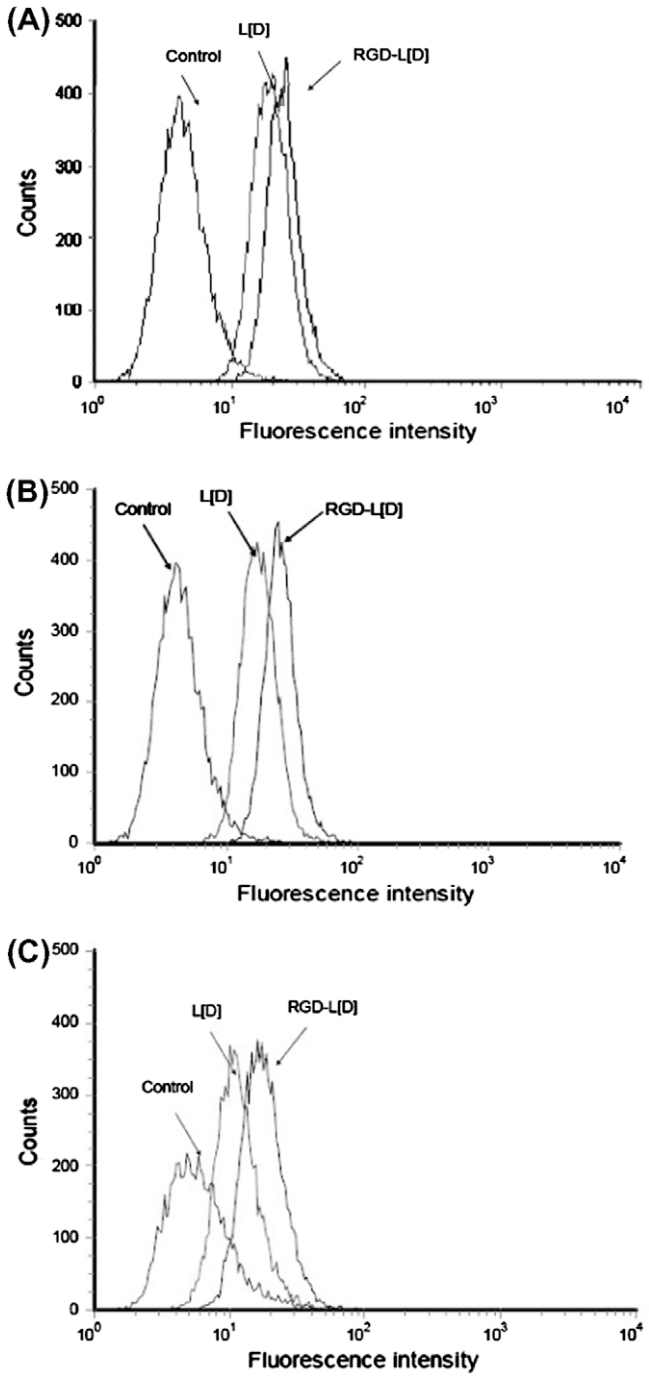

Fig. 4. Intracellular Dox fluorescence intensity in melanoma B16 (A), B16F10 (B) and HUVEC cells (C) treated with L[D] and RGD-L[D] (containing 40 μg Dox/ml) at 37 °C for 1 h.

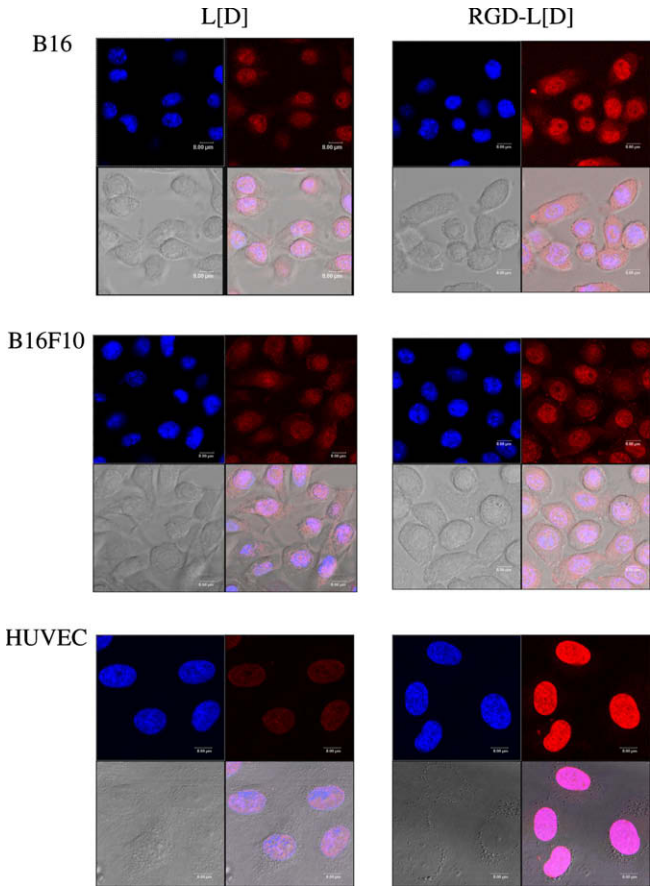

Fig. 5. Confocal microscopy images of melanoma cells B16, B16F10 and HUVEC cells incubated with L[D], RGD-L[D] for 3 h at 37 °C. Dox concentration was 40 μg/mL. Cells were fixed and then treated with Hoechst 33258 (blue) for nuclei staining. Red: fluorescence of Dox. Blue: fluorescence of Hoechst. Pink: the merged fluorescence of blue and red, produced by the co-localization of Hoechst and Dox. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

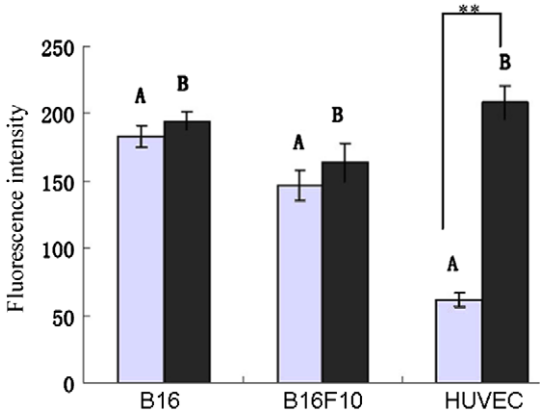

Fig. 6. Fluorescence intensity of doxorubicin after incubating with L[D] (A), RGD-L[D] (B) for 3 h at 37 °C. Dox concentration was 40 μg/mL. (**, significant difference, $P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Intracellular distribution of DOX

Cellular uptake and distribution of Dox in the three cell lines were also investigated by laser confocal scanning microscopy (LCSM). Fig. 5 showed that greater Dox fluorescence intensity was observed in cells treated with RGD-L[D] compared to those treated with L[D]. Majority of Dox fluorescence was observed in the nuclear region. This result validated that the RGD ligand promotes Dox intracellular endocytosis in receptor-mediated manner [14]. Quantitative studies of fluorescence intensity were shown in Fig. 6. Cells were treated for 3 h with RGD-L[D] and L[D], respectively. RGD-coupling of liposomes significantly increased the average fluorescence intensity for cell lines, especially for HUVECs. These data agreed with all the confocal microscopy imaging results (Fig. 5).

3.5. In vitro cytotoxicity assay

The cytotoxicity of different liposomes to B16F10 cells was performed, and 50% inhibition concentration (IC_{50}) was determined from concentration-dependent cell viability curves. The RGD-modified liposomes showed significantly higher level of cytotoxicity compared to other non-targeting liposomes (Table 2).

3.6. In vivo therapeutic efficacy

Fig. 7 shows the results of in vivo therapeutic studies. Compared with L[CD] treatment, RGD-L[CD] dramatically reduced tumor growth ($P < 0.05$), which demonstrated the efficacy of RGD peptide conjugation. RGD-L[CD] group also showed enhanced tumor regression effect compared with RGD-L[D] ($P < 0.001$), and RGD-L[C] ($P < 0.005$), indicating that both CA-4 and Dox play an essential role in the combined therapy. In addition, all of the treatment groups lead to significant anti-tumor effect compared to control group ($P < 0.001$) except RGD-L[D], which may due to the small dose of Dox in our study. No mice died during the treatment.

Little systematic toxicity of various treatments was observed judging from the reduction in mouse net body weights at the end of treatment [15]. The net body weights increased slightly after treatments with RGD-L[CD] and L[CD] compared with RGD-L[D] and RGD-L[C] groups, which showed similar body weight loss as that in physiological saline control group.

Table 2
Cytotoxicities of various liposomal formulations against melanoma B16F10 in vitro ($n = 3$).

Formulations	IC_{50} on B16F10 cells, μM
Free doxorubicin	0.11 ± 0.01
L[D]	0.34 ± 0.11
L[C]	0.21 ± 0.01
L[CD]	0.10 ± 0.03[a]
RGD-L[CD]	0.06 ± 0.01[b]

[a] $P < 0.01$, *vs.* L[C] and L[D].
[b] $P < 0.01$, *vs.* L[CD].

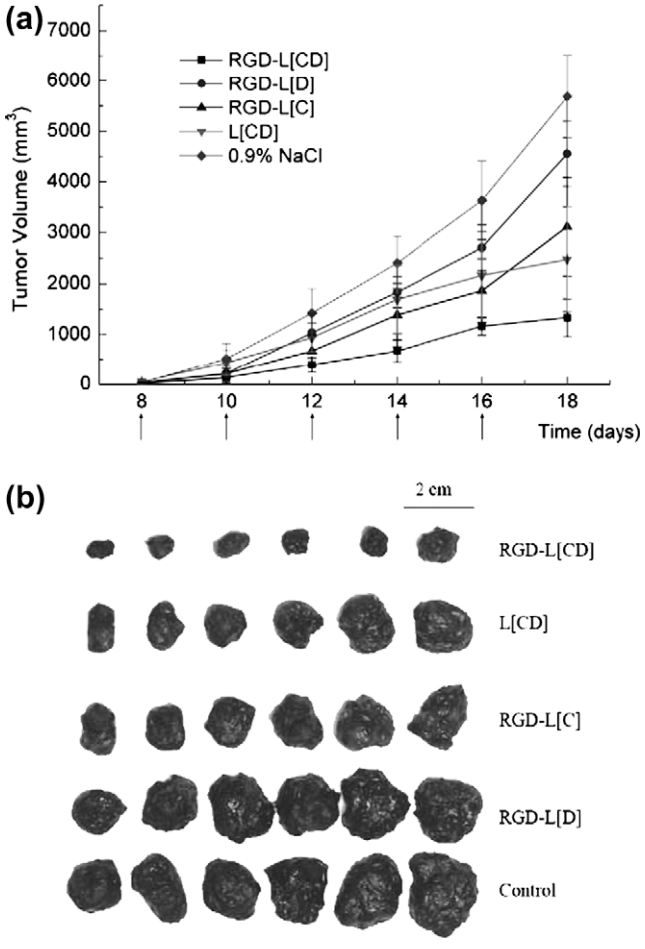

Fig. 7. Tumor growth inhibition by multiple-dose administration of various liposomes in C57BL/6 mice bearing B16F10 tumor. Mice were treated with i.v. injection of RGD-L[CD], RGD-L[D], RGD-L[C] and L[CD] at the doses of 25 mg CA-4/kg and 0.8 mg Dox/kg, with saline as a control. Tumor size was measured for each animal every other day starting from the initial treatment ($n = 6$). Results are expressed as means ± SD. Arrows represent drug administration.

4. Discussion

Increased expression of $\alpha_V\beta_3$ integrins was seen in melanoma cells [16]. In addition, endothelial cells in tumor blood vessels also overexpress α_V integrins [17]. The RGD peptide specifically binds to the $\alpha_V\beta_3$ integrins on melanoma tumor cells and endothelial cells. Due to their significant role in tumor growth and metastasis, those two types of cells have been shown to be promising targets for cancer treatment.

Up to now, few studies have been reported on co-delivery of multiple agents in liposomes. This is probably due to difficulties associated with the efficient and stable encapsulation of two drugs in the same liposome, as well as the challenges in controlling the release rate of chemically disparate drugs within one liposome composition [18]. In this study, two different methods were chosen for the respective loading of CA-4 and Dox into liposomes based on their chemical properties to achieve favorable loading efficiency, stability and drug release rate. CA-4 is a tubulin-binding vascular disrupting agent with a wide therapeutic window, which exhibits a selective toxicity for proliferating endothelial cells in vitro and induces vascular shutdown by disrupting the cytoskeletal structures in tumor models in vivo[10]. It was reported that at doses of 30 and 10 mg/kg CA-4P (prodrug of CA-4) reduced blood flow rate of the P22 rat tumor by 100 fold at 6 h after treatment, and blood flow partially or completely recovered by 24 h [19], which facilitates the access of next dose. We choose CA-4 in our study as it is lipophilic and can be easily incorporated into the lipid bilayer membrane during the preparation of liposomes. Doxorubicin is a cytotoxic agent which has been widely used in clinical cancer therapy. Ammonium sulfate gradient remote-loading method is robust to attain high encapsulation efficiency and slow release rate of doxorubicin. Thus, co-encapsulation of CA-4 and Dox is easily realized. The dose and schedule were chosen according to published literatures [19,20], which is safe and effective.

In our study, preparation method of drug delivery system was specifically designed for the therapeutic rationale. Apparently, the optimal scheduling of vascular disrupting agents (VDAs) and chemotherapeutic agents are vital for the therapeutic effect and toxicity in combination therapy. When the two drugs are given separately, the additive response, which was the best outcome in most studies, was only obtained by administering the VDAs within a few hours after giving the chemotherapeutic drug [2]. In our experiments, however, optimally designed drug carrier (RGD-modified liposomes) brought a greater tumor response in vivo. The benefit was owing to the prolonged release profile of doxorubicin compared to CA-4, which resulted from different drug loading method. Loaded by ammonium sulfate gradient method [7], doxorubicin is present in an aggregated gel-like state at the inner phase of liposomes, which leads to slow dissolving and releasing. On the contrary, CA-4 is dissolved in the lipid membrane and can be read-

ily released from liposomes [11]. The benefit from the differential drug release rates of CA-4 and Dox in our combination therapy might be extended to a wide range of other therapeutic agents.

Reduction in dose-limited toxicity should be one of the major therapeutic benefits of this combination therapy. The result of in vivo experiment proved that the combined VDA-chemotherapy treatment did not aggravate body weight loss compared to either CA-4 liposomes or doxorubicin liposomes alone. Several pre-clinical studies also showed greater anticancer effects without associated increase in toxicity [21–25] or cytotoxic agent-specific normal tissue damage [21,26].

It has been demonstrated in in vivo experiments that the liposomes loaded with both CA-4 and Dox effectively delayed the growth of subcutaneous B16F10 xenograft tumors in C57BL6 mice much more than liposomes loaded with either drug alone, which exhibits synergistic effect of the combined therapeutics. This phenomenon might be due to a unique mechanism in our combination therapy, i.e., entrapment of doxorubicin in the tumor tissue through CA-4-induced vascular shutdown [4]. We hypothesize that CA-4 from the liposomes firstly disrupted the vascular matrix, leading to entrapment of the liposomal Dox inside the tumor site, the Dox was then released subsequently to exert its cytotoxicity. Although further experiments are needed to validate this hypothesis, it is clear that understanding the physiological process after drug administration in vivo would be the key step towards improvement and application of this strategy.

Despite some reported studies on combining antivascular agent with cytotoxic agent, there has been little study focused on the interaction between the two drugs on PK/PD. Further studies should address optimal dose and ratio of the two drugs to further improve the therapeutic index.

In conclusion, CA-4 and doxorubicin were co-encapsulated in liposomes for sequential release, and RGD peptide was attached on the surface of liposomes to facilitate intracellular uptake of liposomal drugs. Both of the factors strongly correlated with an increased anti-tumor response. We believe that this drug delivery system for integrated combination therapy may lead to the development of more effective and less toxic cancer treatments, especially for intractable tumor diseases.

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