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Research paper

Sequential treatment of drug-resistant tumors with RGD-modified liposomes containing siRNA or doxorubicin

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ABSTRACT

Tumor targeting drug delivery systems are being the ideal carriers of systemic administration for tumor therapy. We have reported previously that RGD peptide (arginine-glycine-aspartic acid)-modified liposomes containing drugs could increase targeting to tumor by binding with the integrin receptors overexpressed on tumor cells. RNA interference plays an important role on down-regulation of P-glycoprotein (P-gp), which is a drug efflux transporter overexpressed on multi-drug-resistant (MDR) tumor cells. To improve MDR tumor therapy, sequential treatment strategy with RGD-modified liposomes containing P-gp targeted small interference (siRNA) or doxorubicin (DOX) was reported in this study. When targeted via RGD to tumor-cell-surface and tumor neovasculature endothelial cell receptors, cationic liposomes could specifically deliver siRNAs to tumor cells and thus reverse drug resistance by down-regulation of P-gp, following administration of targeted liposomes containing DOX that inhibit formerly drug-resistant tumors. From the current results, the combination use of DOX and P-gp targeted siRNA showed significantly higher in vitro cytotoxicity in tumor cells than liposomal DOX alone. In vivo studies in a mouse model of drug-resistant MCF7/A tumor demonstrated significantly greater inhibition of tumor growth followed by the sequential treatment of RGD-modified liposomes containing siRNA or DOX when compared to liposomal DOX alone. Also, ex vivo tissue imaging studies have shown the accumulation of siRNA and DOX in tumors at same site-specific manner. These results suggested that the sequential treatment of P-gp gene silencing and cytotoxic drug with RGD-modified liposome drug delivery system could be a promising clinical treatment for drug-resistant tumors.

1. Introduction

Multi-drug resistance (MDR) is one of the major obstacles in cancer chemotherapy. P-glycoprotein (P-gp), encoded with MDR1 gene, is an energy-dependent efflux transporter and plays an important role in decreased intracellular accumulation and cytotoxic effect of anticancer drugs in drug-resistant tumors [1,2]. In the past decades, many small-molecule organic compounds were used as functional inhibitors, such as verapamil [3,4], cyclosporine A [5] and Valspodar (PSC833) [6], which have shown potent effects on modulation of P-gp activity in early clinical trials, but their long-term safety and unpredicted pharmacokinetic interaction with anticancer drugs and other transport proteins are still to be elucidated.

A novel strategy to overcome drug resistance is to use RNA interference (RNAi) to silence the expression of the efflux transporter. RNAi is a post-transcriptional gene silencing mechanism mediated by small interfering RNA (siRNA), a 21–25 nucleotide (nt) double-stranded RNA molecule, which could be incorporated into RNA-induced silencing complex (RISC) and induces degradation of target mRNAs in a sequence-specific manner [7]. Previous studies have shown that RNAi could reverse the MDR phenotype selectively and successfully [8–10]. Compared to those chemical functional inhibitors, more potential, specific and powerful inhibition of P-gp expression would be conducted by MDR1 gene silencing triggered by siRNA.

However, as a negative-charged and water-soluble macromolecule, in vivo application of free siRNA faces many barriers such as ribonuclease (RNase) degradation, elimination, poor permeability and endosomal trapping [7]. Cationic liposomes were widely used as a non-viral vector for in vitro and in vivo siRNA delivery application [11,12]. Liposome-siRNA complex (lipoplex) was formed by the electrostatic interaction of cationic liposome with siRNA based on their opposite surface charges [13]. It has been shown that the resulting lipoplexes could efficiently protect siRNA from RNase degradation and improve cell uptake by endocytosis.

To improve therapeutic index of drug-resistant tumor, many strategies involving combination use of anticancer drug and P-gp

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inhibitor (functional inhibitors or siRNA) have been reported. In our previous studies, verapamil, as a functional inhibitor of P-gp efflux activity, was incorporated into the liposomes simultaneously containing doxorubicin for treatment of MDR tumor cells[3]. With RNAi technology developed in recent years, simultaneous delivery of anticancer drug and P-gp targeted siRNAs with liposomes or nanoparticles were also reported as new strategies for MDR cancer therapy [14–16]. These results suggested that the combination use of P-gp gene silencing and cytotoxic drug using a suitable carrier can overcome tumor drug resistance. However, two deficiencies of dual agents co-delivery systems should be also considered. First, the combination in a single liposome drug delivery system of siRNA and anticancer drug might be difficult in dosage fractionation when used in clinic. Second, sufficient time should be allowed for achieving substantial down-regulation of P-gp expression after administration of siRNA-lipoplex formulation [17].

In recent years, RGD-based liposomal delivery strategies were newly developed in our research group [18,19]. It has been shown that RGD-based liposomes could be a promising drug delivery system for targeting tumor therapy via RGD peptide specifically targeting to integrin receptors overexpressed on tumor-cell-surface and tumor neovasculature endothelial cells.

Therefore, we hypothesized that sequential administration of dual agents using RGD-modified liposomes would result in increased therapeutic efficacy of MDR tumors by means of first substantial down-regulation of the P-gp involving RGD-mediated targeting delivery of MDR1-siRNA and following a second wave of therapy involving intravenous (*i.v.*) administration of RGD-modified liposomes containing doxorubicin.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC), dioleoyl-trimethylammonium-propane (DOTAP), cholesterol and dioleoyl-phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA); distearoylphosphatidylethanolamine with covalently linked polyethylene glycol of molecular weight 2000 (DSPE-PEG_{2000}) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene-glycol)]-hydroxy succinamide, PEG Mw 2000] (DSPE-PEG_{2000}-NHS) were purchased from NOF Co. (Tokyo, Japan); doxorubicin hydrochloride (DOX) was kindly provided as a gift by Haizheng Pharmaceutical Co. (Zhejiang, China), Arginine-glycine-aspartic acid (RGD) was obtained from (Zhongkeyaguang Biotechnology Co., Ltd., Beijing, China), Hoechst 33258 was purchased from Molecular Probes Inc. (Oregon, USA), OPTI-MEM was purchased from Invitrogen (NY, USA); DSPE-PEG_{2000}-RGD was synthesized according to previously reported method[18].

Small interference RNA (sense strand: 5′-GAA ACC AAC UGU CAG UGU AdTdT; antisense strand: 5′-UAC ACU GAC AGU UGG UUU CdTdT) [20] targeted to the human MDR1 mRNA was synthesized and purified with HPLC by GenePharma Co. Ltd (Shanghai, China). FAM-siRNA and scramble siRNA were also obtained from GenePharma (Shanghai, China).

2.2. Cell line and culture

DOX-resistant human breast cancer MCF7/A cells were obtained from the Institute of Hematology & Blood Diseases Hospital (Tianjin, China). The cells were maintained in MEM medium (Macgene, Beijing, China) supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified atmosphere containing 5% CO_2. All experiments were performed on cells in the exponential growth phase.

2.3. Preparation of siRNA-lipoplex and liposomal DOX

Lipid compositions of DOTAP:Chol:DOPE (25:30:43, mol/mol), DOTAP:Chol:DOPE:DSPE-PEG_{2000} (25:30:43:2 or 25:30:43:4 mol/mol), and DOTAP:Chol:DOPE:DSPE-PEG_{2000}:DSPE-PEG_{2000}-RGD (25:30:43:1:1 or 25:30:43:2:2, mol/mol) were used for the preparation of unmodified cationic liposomes (named as Lipo), sterically stabilized cationic liposomes (named as 2% PEG-Lipo or 4% PEG-Lipo) and RGD-modified cationic liposomes (named as 1% RGD-Lipo or 2% RGD-Lipo), respectively. The liposomes were prepared as reported in previous study [21]. Briefly, lipids were dissolved in chloroform–methanol (2:1, v/v), and the lipid film formed under rotary evaporation was hydrated with sterilized 5% glucose solution, and after sonication, additional extrusion with 0.1 μm pore polycarbonate membrane filter was processed for 10 cycles to obtain the optimal size distribution of liposomes. The siRNA-lipoplexes (Lipo-siRNA) were prepared by mixing the liposome suspensions with siRNA in sterilized 5% glucose solution with the charge ratio of 4:1(+/−).

RGD-modified sterically stabilized liposomes (RGD-SSL) composed of EPC, Chol, PEG-DSPE and RGD-PEG-DSPE (20:10:1:1, mol/mol) were prepared by the method described previously [19], and the remote-loading method with an ammonium sulfate gradient was used for loading DOX into liposomes (RGD-SSL-DOX). In brief, the lipid film was hydrated with 123 mM ammonium sulfate solution, and after sonication, the liposomes were passed though a Sephadex G50 (Phamacia Biotech, NJ, USA) gel-filtration column pre-equilibrated in 5% glucose solution to exchange the external phase. Then, the liposomes with an ammonium sulfate gradient were incubated with a proper amount of DOX at 50 °C for 10 min, and DOX was loaded into the inner phase of liposomes gradually. Free DOX was then removed by gel-filtration.

The particle size and zeta potential of the prepared liposomes were measured using dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS, Malven, UK). The analysis was performed with 10mW He–Ne laser (633 nm) at scattering angle of 90° at 25 °C. The results were determined three times for each sample.

2.4. In vitro siRNA transfection

MCF7/A cells were seeded 3.5×10^5 per well in six-well plates. After 24-h proliferation, various liposome formulations containing FAM-siRNA at the final concentration of 50 nM, including Lipo-siRNA, 2% PEG-lipo-siRNA, 4% PEG-lipo-siRNA, 1% RGD-lipo-siRNA and 2% RGD-lipo-siRNA were exposed to cells, respectively, and incubated for additional 6 h at 37 °C in humidified air with 5% CO_2. After incubation, the cells were harvested and washed three times with pre-cooled phosphate buffer solution (PBS) and then analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) immediately. Free FAM-siRNA was used as a control.

2.5. Intracellular accumulation of doxorubicin

MCF7/A cells were seeded 1×10^5 per well in six-well plates. After 24-h incubation, cells were transfected with 50 nM siRNA-lipoplexes in OPTI-MEM medium and incubated for 6 h, and then the OPTI-MEM medium was replaced with the fresh RPMI-1640 medium containing 10% FBS, and cells were incubated for another 48 h. After that, free DOX (40 μg/ml) was added into each well and incubated for additional 1 h at 37 °C. Finally, cells were harvested and washed twice with ice-cold PBS and then analyzed on a flow cytometer immediately.

2.6. In vitro cytotoxicity

In vitro cytotoxicities of the sequential treatment of Lipo-siRNA and DOX on drug-resistant MCF7/A cells were performed by

sulforhodamine B (SRB) assay [22]. Briefly, Aliquots of 3×10^5 MCF7/A cells were seeded in 60-mm dishes. After 24-h proliferation, the cells were transfected with RGD-lipo-siRNA in OPTI-MEM medium without antibiotics and serum. The final concentration of siRNA was 50 nM. After 6-h transfection, the culture medium was refreshed with RPMI-1640 medium supplemented with 10% FBS and 50 U/ml penicillin and 50 μg/ml streptomycin. After another 42-h incubation, cells were harvested and seeded in 96-well plates at a density of 5000 cells per well. After 24-h proliferation, cells were treated with a series of concentrations of DOX for 48 h. Then, the culture medium was carefully removed, and cellular protein was fixed by the addition of 10% TCA (trichloroacetic acid) at 4 °C for 1 h, following the 96-well plates were washed by five washing cycles using deionized water and air-dried. SRB solution was added into each well and allowed for a 15-min staining. Then, SRB solution was removed, and plates were washed five cycles using 1% acetic acid. After air-dried, the 10 mM Tris base solution was added into the 96-well plates to solubilize the protein-bound dye on a gyratory shaker for 15 min. The absorbance values were read on a microplate reader (BIO-RAD model 680, Bio-Rad Laboratories, Inc. Shanghai, China) at the wavelength of 540 nm, and then the cytotoxicity (IC_{50}) was determined by the method described in the SRB assay.

2.7. Intracellular localization of DOX and siRNA

Cellular internalization of DOX and siRNA in MCF7/A cells was monitored by confocal microscopy. Aliquots of 12×10^4 cells were seeded in 35-mm dishes with a glass cover slip at the bottoms. After 24-h proliferation, cells were transfected with 1% RGD-lipo-siRNA in OPTI-MEM medium following with the addition of RGD-SSL-DOX (40 μg/ml). The final concentration of FAM-siRNA was 100 nM. After 6-h incubation, cells were washed with cooled PBS immediately and fixed by histiocyte stationary liquid (Saichi Biotech, Beijing, China) at room temperature for 10 min, followed by cell nuclei staining with Hoechst 33258 for 15 min before washed three times with PBS for confocal microscopy analysis. Leica SP5 confocal microscope (Heidelberg, Germany) was used to observe cellular localization of DOX (λ_{ex} 480 nm/λ_{em} 540 nm) and FAM-siRNA (λ_{ex} 488 nm/λ_{em} 518 nm).

2.8. In vivo tumor growth inhibition study

Female Balb/c mice (6–8 weeks old), weighing 18–22 g were purchased from 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 of Peking University. Each of 5×10^6 MCF7/A cells was inoculated subcutaneously in the right flank of the Balb/c mice [23]. When tumor size reached 120–150 mm^3 in volume, animals were sacrificed, and tumors were aseptically dissected and minced with scissors into ~15-mm^3 pieces in the sterile physiological saline, after which tumor tissues were transplanted s.c. into the armpit of the mice. Once tumor size was ~150 mm^3 in volume, mice were randomly assigned to treatment groups (5–6 animals per group). Four doses of Lipo-siRNA (2 mg/kg) were administered intravenously via tail vein at the 11th, 13th, 15th, 17th day after inoculation, and following the administration of RGD-SSL-DOX (4 mg/kg) at the 13th, 15th, 17th day, and the last injection of RGD-SSL-DOX was performed on the 19th day after inoculation. Seven formulations, including 5% glucose, blank liposomes (RGD-SSL plus RGD-Lipo), RGD-SSL-DOX, RGD-SSL-DOX plus Lipo-siRNA(scramble), RGD-SSL-DOX plus 2% PEG-Lipo-siRNA(MDR1), RGD-SSL-DOX plus 1% RGD-Lipo-siRNA(MDR1) and RGD-SSL-DOX plus 2% RGD-Lipo-siRNA(MDR1), were given into Balb/c mice via tail vein, respectively. After the final administration of DOX formulation, the mice were further observed for 1 week and then sacrificed on the 27th day of the whole. Body weight and tumor size of each mice were measured once daily. Tumor volumes were calculated using the formula: volume = length × $width^2/2$ [24].

2.9. Immunofluorescence detection of P-gp

After the tumor inhibition study, tumor tissues were harvested, immediately placed in Tissue-Tek OCT embedding medium (Sakura Finetek, Tokyo, Japan), and frozen on dry ice, then cut into 5-μm cryosections. Sections of tumor tissue were fixed in pre-cooled acetone for 10 min at 4 °C. The sections were incubated with 1% BSA for 30 min at room temperature and then with 1:100 anti-P-gp mouse monoclonal antibodies (Calbiochem, Germany) overnight at 4 °C. After washed with PBS, the sections were incubated with 1:100 FITC-labeled goat anti-mouse IgG (Genetex, San Antonio, TX) at 37 °C for 1 h, stained with Hoechst33258 and mounted. Leica SP5 confocal fluorescence microscope (Heidelberg, Germany) was used for photograph [25].

2.10. Western blotting analysis of P-gp

The protein was extracted from tumor tissues as directed by the instructions of Eukaryotic Membrane Protein Extraction Reagent Kit (Keygen Biotech, Nanjing, China). The samples containing equivalent amounts of protein (30 μg) were applied to 7.5% acrylamide denaturing gels (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The separated proteins were transferred onto nitrocellulose membranes overnight for 70 min at 4 °C. Blotting membranes were incubated with blocking solution [3% BSA dissolved in Tris-buffered saline Tween-20 (TBST) buffer (pH 7.5, 10 mmol/l Tris–HCl, 150 mmol/l NaCl, and 0.1% Tween-20)] overnight at 4 °C, washed three times and then were incubated with mouse anti-P-glycoprotein (1:1000; Calbiochem, Germany) in TBST for 2 h at room temperature. Internal control was carried out using GAPDH antibody (1:2000; Sigma–Aldrich, St. Louis, MO, USA). After several washes with TBST buffer, the membranes were incubated for 2 h with goat anti-mouse IgG-HRP antibody (1:2000; Santa Cruz Biotechnology). The membranes were then processed with enhanced chemiluminescence Western blotting detection reagents (Millipore Corporation, Billerica, USA).

2.11. Ex vivo tissue imaging

To investigate tumor targeting effect of RGD-modified liposomes, Kodak in vivo imaging system (Imaging Station IS2000MM, Kodak, USA) was used to observe the in vivo distribution of siRNA and DOX in the Balb/c mice xenografted MCF7/A tumors. When tumor size reached ~300 mm^3, two groups of mice were given respectively with the formulations of 2% PEG-lipo-siRNA plus SSL-DOX and 1% RGD-lipo-siRNA plus RGD-SSL-DOX at the concentrations of FAM-siRNA, 2 mg/kg and DOX, 10 mg/kg via tail vein according to the administration approach of tumor inhibition experiments. At 4-h post-injection, the mice were sacrificed, and then tissues were excised and imaged with appropriate wavelength (FAM-siRNA: λ_{ex}: 490 nm, λ_{em}: 540 nm; DOX: λ_{ex}: 480 nm, λ_{em}: 590 nm)[26]. Images were analyzed using the imaging station IS2000MM software.

2.12. Statistical analysis

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 very 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).

3. Results

3.1. Physicochemical characterization of liposomes

The average size and zeta potential of different formulations including Lipo, PEG-Lipo (2% or 4%), RGD-Lipo (1% or 2%) and RGD-Lipo-siRNA (1% or 2%) were measured. As shown in Table 1, average sizes of the prepared liposomes were all less than 200 nm. Compared to blank Lipo, particle size of PEG-Lipo and RGD-Lipo could be significantly increased with the PEG- or RGD modification, but zeta potential was decreased with the ratio of DSPE-PEG increasing. Furthermore, siRNA binding would induce further increase of particle size of siRNA-lipoplex that was about 160 nm.

3.2. Cellular uptake of siRNA

As shown in Fig. 1, the sequence of fluorescence intensity in cells after treated with various siRNA formulations was as follows: free siRNA < Lipo-siRNA < 4% PEG-lipo-siRNA < 2% RGD-lipo-siRNA < 2% PEG-lipo-siRNA < 1% RGD-lipo-siRNA. It was suggested from this result that cationic liposomes could significantly enhance siRNA translocation into cells when compared to that of negative-charged free siRNA, also, PEG-modified or RGD-modified on the surface of liposomes could obtain further enhancement of siRNA transfection. Interestingly, lesser siRNA transfection was found in the liposomes with higher ratio of PEG modification or RGD modification. Under the equal molar ratio of PEGylation (equivalent to 2% or 4% mol ratio of PEG-DSPE), RGD-modified lipoplex seemed to display more enhancement of siRNA transfection than non-targeted lipoplex.

Table 1
Average size, polydispersity and zeta potential of various formulations ($n = 3$).

Sample	Size/nm	Polydispersity	Zeta potential/mV
RGD-SSL-DOX	96.1 ± 1.6	0.226 ± 0.044	-9.6 ± 1.9
Lipo	70.0 ± 7.0	0.233 ± 0.047	52.5 ± 2.9
2% PEG-Lipo	93.1 ± 3.6	0.222 ± 0.019	39.9 ± 3.9
4% PEG-Lipo	95.7 ± 12.4	0.201 ± 0.004	37.0 ± 4.2
1% RGD-Lipo	97.9 ± 12.6	0.228 ± 0.011	37.6 ± 5.1
2% RGD-Lipo	102.0 ± 12.3	0.201 ± 0.021	35.9 ± 0.2
1% RGD-Lipo-siRNA	160.2 ± 6.3	0.211 ± 0.038	21.1 ± 1.7
2% RGD-Lipo-siRNA	168.6 ± 16.9	0.220 ± 0.040	18.2 ± 1.5

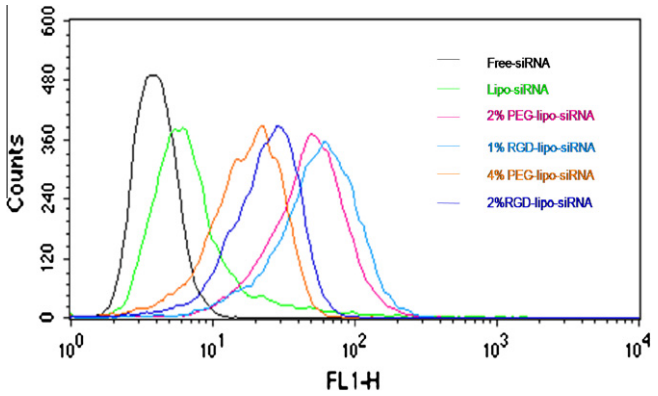

Fig. 1. Fluorescence intensity of FAM-siRNA uptaken by MCF7/A cells. Cells were transfected with free siRNA, Lipo-siRNA, 2% PEG-lipo-siRNA, 4% PEG-lipo-siRNA, 1% RGD-lipo-siRNA, 2% RGD-lipo-siRNA at 37 °C for 6 h, the final concentration of siRNA was 50 nM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

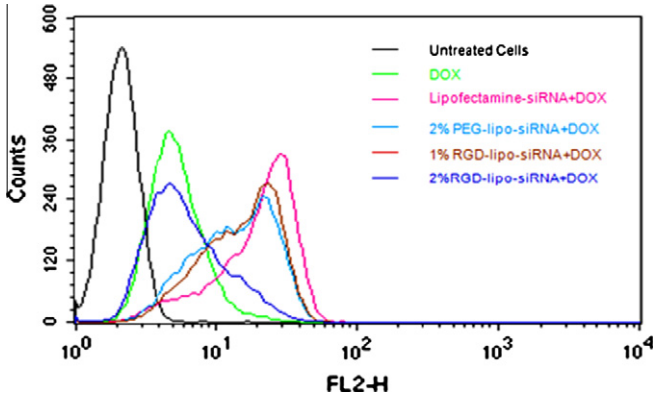

Fig. 2. Accumulation of doxorubicin in MCF7/A cells measured by flow cytometry. Cells were first transfected with MDR1 siRNA (50 nM) formulated in lipofectamine, 2% PEG-lipo, 1% RGD-lipo, 2% RGD-lipo for 48 h, and then cells were incubated with doxorucin (40 μg/ml) for 1 h at 37 °C. Cells incubated with doxorubicin alone without additional siRNA transfection were used as negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

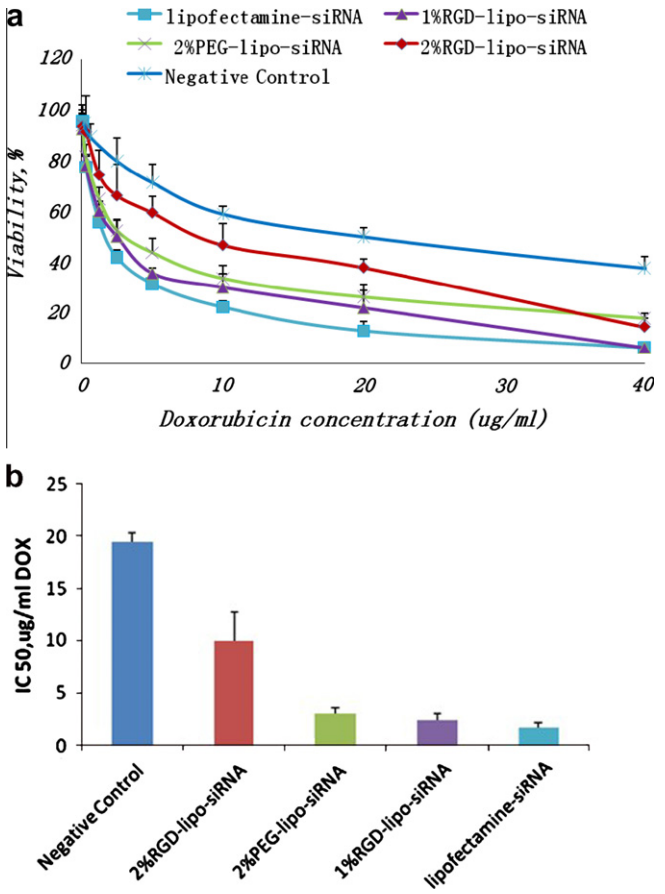

Fig. 3. Cytotoxicity of doxorubicin on MCF7/A cells after 48-h incubation with different formulations: lipofectamine, 2% PEG-lipo, 1% RGD-lipo, 2% RGD-lipo. (a) Dose–response curves of doxorubicin on cell viability; (b) IC_{50} of doxorubicin on cells treated with different formulations. MCF7/A cells treated with doxorubicin without additional siRNA transfection were used as negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Intracellular accumulation of doxorubicin

To improve intracellular accumulation of DOX, drug resistance-related P-gp should be interfered by MDR1 gene-related siRNA. The

fluorescence intensity of intracellular DOX after treated with various different siRNA formulations were determined by flow cytometer assay and shown in Fig. 2. It was proved from this result that combination use of siRNA formulations could significantly enhance intracellular accumulation of DOX in DOX-resistant MCF7/A cells. Lipofectamine, as a positive transfection agent, has shown the selected siRNA sequence was effective in MDR1 gene silencing. Similar as the results of siRNA transfection, the sequence of intracellular DOX accumulation after treated with different siRNA-lipoplex formulations were as follows: 2% RGD-lipo-siRNA < 2% PEG-lipo-siRNA < 1% RGD-lipo-siRNA.

3.4. In vitro cytotoxicity

Dose–response curves of DOX cytotoxicity against MCF7/A cells after treated with different siRNA-lipoplex formulations was performed by SRB assay and shown in Fig. 3a. As shown in Fig. 3b, differences in cytotoxicities (IC_{50}) of DOX against MCF7/A cells after treated with different siRNA-lipoplex formulations were obviously observed as the following rate: Negative Control (19.49 ± 0.81 μg/ml) > 2% RGD-lipo-siRNA (9.96 ± 2.78 μg/ml) > 2% PEG-lipo-siRNA (3.03 ± 0.55 μg/ml) > 1% RGD-lipo-siRNA (2.44 ± 0.62 μg/ml) > Lipofectamine-siRNA (1.73 ± 0.44 μg/ml). Cytotoxicity of blank vehicles was subtracted as background.

3.5. Intracellular localization of siRNA and DOX

Intracellular localization of FAM-siRNA (green) and DOX (red) was investigated using laser confocal microscope and shown in Fig. 4. After 6-h incubation, FAM-siRNA was observed in cytoplasm with a relative uniform distribution, and DOX was found mainly in nuclear region.

3.6. Evaluation of in vivo tumor inhibition and safety

Fig. 5a shows tumor therapeutic efficacy of various formulations composed of DOX and/or siRNA on Balb/c mice xenografted DOX-resistant MCF7/A tumors. No effect on tumor growth was found in the group that treated with blank liposomes. Interestingly, it was consistent with in vitro cytotoxicity that the optimal tumor growth inhibition effect was observed in the animals that treated with sequential multi-dose treatment of 1% RGD-Lipo-siRNA (MDR1) and RGD-SSL-DOX via intravenous injection. Compared to the control (5% glucose and blank liposomes), significant inhibition effects of tumor growth were observed in the groups that treated with RGD-SSL-DOX with or without RGD-Lipo-siRNA (MDR1). The similar profiles of tumor growth inhibition between the groups that treated with RGD-SSL-DOX and RGD-SSL-DOX/1% RGD-Lipo-siRNA (scramble) implied that no interference effect on P-gp was shown by scramble siRNA sequence. In comparison with 2% PEG-modified siRNA-lipoplex, the combination use of 2% RGD-modified siRNA-lipoplex did not show any enhancement of tumor inhibition effect of RGD-SSL-DOX formulation, although moderate enhancement was seen when compared to those treated with RGD-SSL-DOX and RGD-SSL-DOX/1% RGD-Lipo-siRNA (scramble).

To evaluate the systemic toxicity of various formulations, the body weight changes in animals were recorded at the same time as tumor size measuring. Seen from Fig. 5b, the body weight changes in animals treated with RGD-SSL-DOX were similarly decreased after four-dose administrations, either with or without combination use of siRNA-lipoplex. It was suggested from this result that the systemic toxicity might be resulted from DOX, but not from siRNA-lipoplex or blank liposomes.

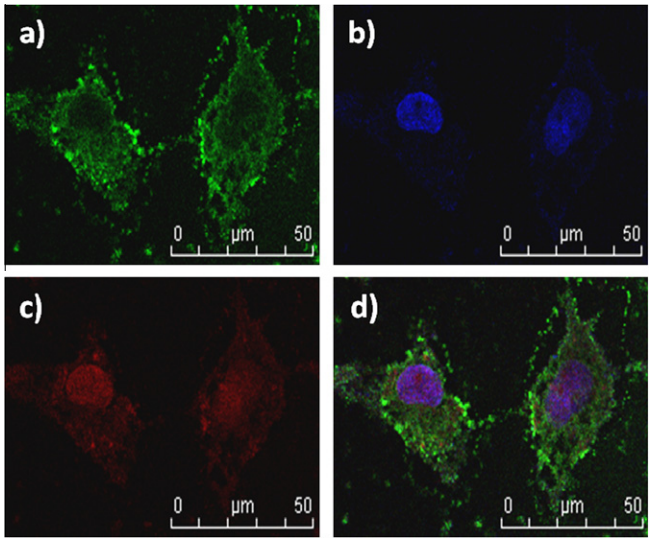

Fig. 4. Intracellular localization of siRNA and doxorubicin in MCF7/A cells detected by confocal microscopy after treated with 1% RGD-Lipo-siRNA (100 nM) and RGD-SSL-DOX (40 μg/ml). (a) FAM-siRNA (green); (b) nucleus stained with Hoechst33258 (blue); (c) doxorubicin (red); (d) merge imaging. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. Immunofluorescence and Western blot analysis of P-gp

To investigate the biological activities of MDR1 siRNA transfected with various formulations, P-glycoprotein expression levels in the tumors were detected by immunofluorescence and Western blot (Fig. 6). Compared to 5% glucose, the Lipo-siRNA (scramble) formulation was rarely found any influence on P-glycoprotein expression; meanwhile, significant influence on P-gp expression was observed from all the Lipo-siRNA(MDR1) formulations, especially from the 1% RGD-Lipo-siRNA(MDR1).

3.8. In vivo distribution of siRNA and DOX

Ex vivo images of tumor and organs that were excised from mice were shown in Fig. 7. In whole-animal imaging, fluorescent signals of FAM-siRNA and DOX in deep organs are often underestimated because of optical impedance by soft tissues. Therefore, ex vivo imaging of tumor and organs was performed immediately at 4-h post-injection. Seen from the images of FAM-siRNA (Fig. 7a) and DOX (Fig. 7b), significant enhancement of tumor targeting was found in in vivo distribution of targeted RGD-modified liposomal siRNA or DOX when compared to that of non-targeted PEG-modified, although both siRNA and DOX were accumulated primarily in tumor and liver as well as lower uptake in other organs.

4. Discussion

During the past decade, RGD peptides have become a popular tool for the targeting of drugs and imaging agents to alphavbeta3-integrin expressing tumor vasculature, and chemical modification have been applied to couple RGD peptides and RGD-mimetics to liposomes, polymers, peptides, small-molecule drugs and radiotracers [27,28]. In recent years, RGD-modified liposomes have been reported as a potential carrier for tumor targeting of anticancer drugs or gene delivery [18,19,29–32]. It was suggested from these results that further enhancement of tumor therapeutic efficacy and minimization of systemic toxicity were achieved by RGD-based strategies.

Up to now, few studies were focused on the combination use of anticancer drugs and siRNA for drug-resistant tumor therapy based

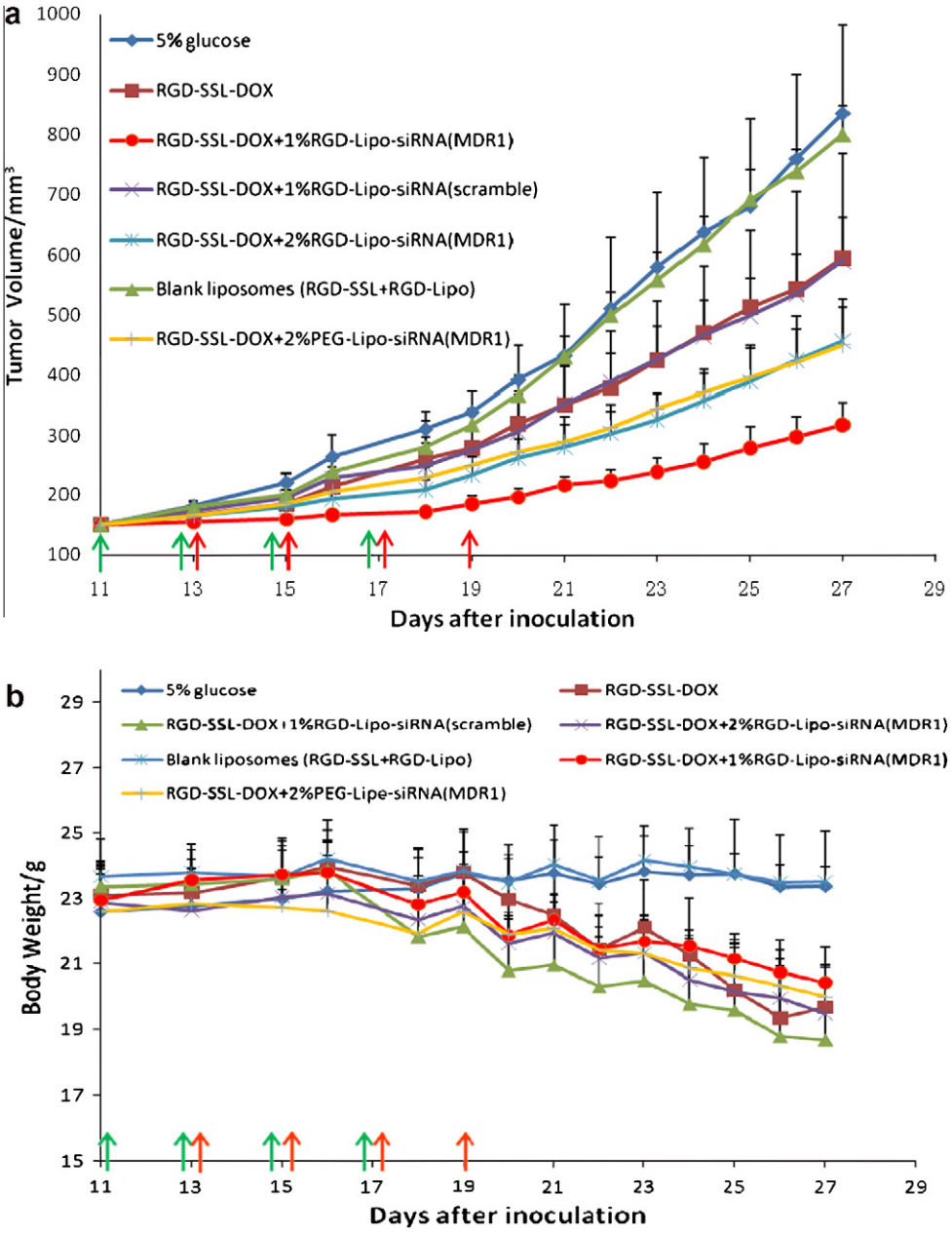

Fig. 5. Evaluation of tumor growth inhibition and systemic safety for co-administration of siRNA-lipoplexes (2 mg/kg) and liposomal DOX (4 mg/kg) on Balb/c nude mice (5–6 animals each group) xenografted drug-resistant MCF7/A tumor. (a) In vivo tumor growth curves and (b) The changes in body weight. Arrows indicate injections of Lipo-siRNA (green) and RGD-SSL-DOX (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on nanoparticulate drug delivery systems, especially for RGD-modified liposomes, although a PEGylated stealth liposome co-encapsulating DOX and verapamil as a functional inhibitor of P-gp had been developed for treatment of DOX-resistant tumor by our research group [3]. Also, RGD-modified cationic liposomes co-encapsulating both DOX and siRNA had been studied but failed in severe aggregation and DOX leakage resulted from unknown interaction of siRNA and DOX-contained cationic liposomes which was prepared by a remote-load method with ammonium sulfate gradient and purified with Sephadex G50 (Pharmacia, USA) gel-filtration (Data not published). Except for this predominant disadvantage, other two deficiencies should be also considered: First, the combination in one liposome drug delivery system of siRNA and DOX might be difficult in dosage fractionation. Second, a sufficient time should be allowed for achieving substantial down-regulation of P-gp expression after administration of siRNA-lipoplex formulation [17]. Therefore, a sequential treatment strategy for drug-resistant tumors with RGD-modified liposomes containing P-gp targeted siRNA or DOX was developed in the present study. It was hypothesized that targeted via RGD to tumor-cell-surface and tumor neovasculature endothelial cell receptors, cationic liposomes could specifically deliver siRNAs to tumor and thus compromise drug resistance by down-regulation of P-gp, following administration of targeted liposomes containing DOX further inhibit the drug sensitivity-enhanced tumors.

Separated preparation of RGD-modified siRNA-Lipoplex and RGD-SSL-DOX was favorable for the stability and the administration of these two drug formulations. The physicochemical characterizations of various formulations were shown in Table 1. RGD-SSL-DOX was well established with about 100-nm particle size and more

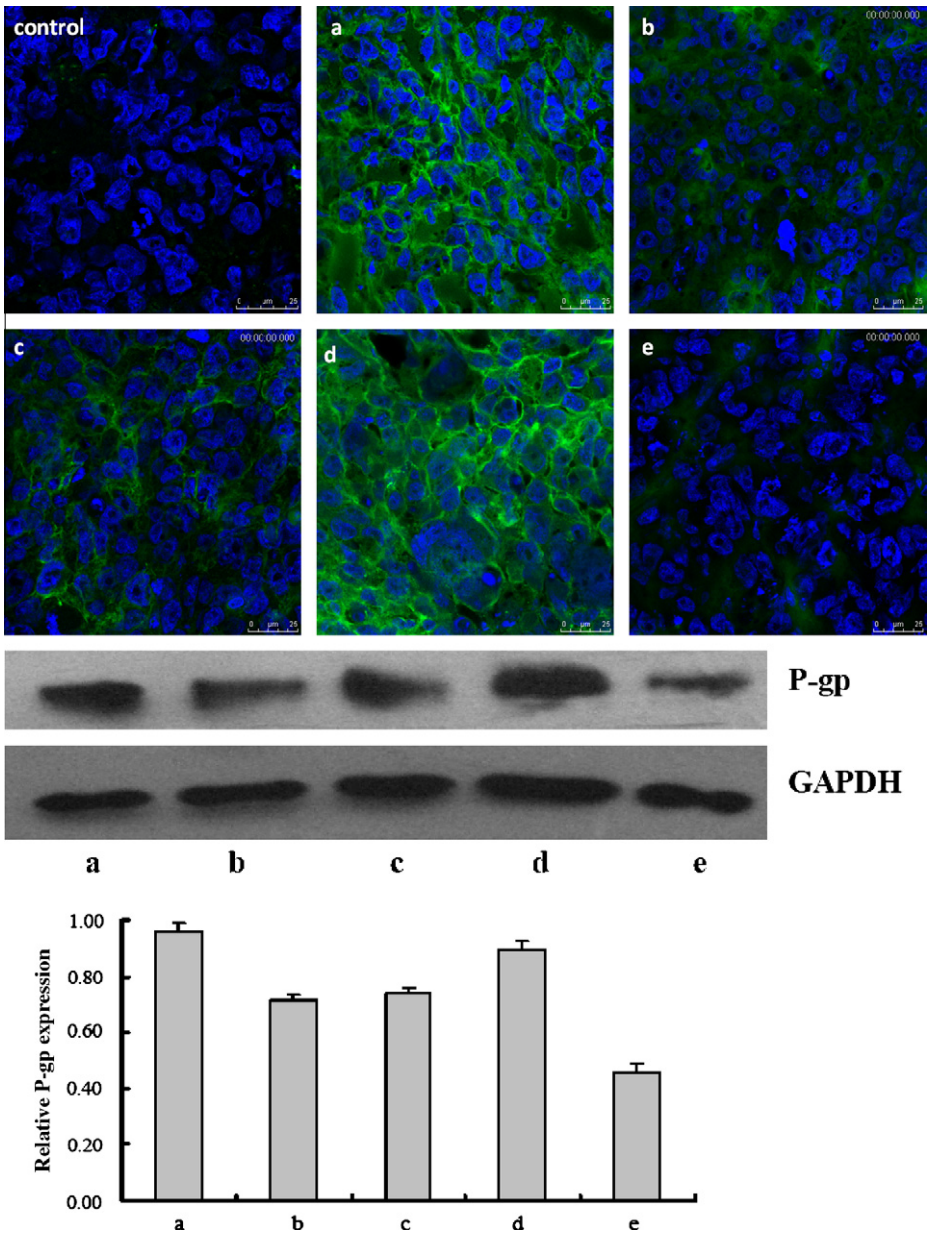

Fig. 6. Detection of P-gp expression in tumors excised from the mice treated with (a) 5% glucose; (b) 2% PEG-Lipo-siRNA(MDR1); (c) 2% RGD-Lipo-siRNA(MDR1); (d) 1% RGD-Lipo-siRNA(scramble); (e) 1% RGD-Lipo-siRNA(MDR1) at the end of tumor inhibition experiments. Left for confocal microscopy, Green color represents for P-glycoprotein binding with FITC-labeled antibody (green), and blue color represents for nuclei stained with Hoechst33258. BSA (1%) instead of FITC-labeled antibody in control. Right for Western blot and the ratio of P-gp expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

than 90% encapsulation efficiency of DOX. Most of siRNA-lipoplexes were of narrow size distributions between 100 nm and 200 nm. Zeta potentials of the lipoplexes were decreased with DSPE-PEG modification, and lower zeta potential was observed in the RGD-Lipo-siRNA liposomes that modified with DSPE-PEG-RGD. As we know, cationic liposomes would have a strong electrostatic interaction with the negatively charged proteins in serum that resulted in aggregation [33], which often caused a reduced transfection efficiency and unpredicted pharmacokinetic properties in vivo. Therefore, the shielding effects of PEGylation on surface positive charge of cationic liposomes would favor the stability of siRNA-lipoplex. In our previous study [34], it was shown that 2% mol PEG-DSPE insertion would significantly enhance the stability of siRNA-lipoplex in the medium containing 50% fetal bovine serum. It was suggested that the hydrophilic long-chain PEG could shield positive charge on the surface of liposomes. The similar aggregation was found among the PEG-Lipo, RGD-Lipo and RGD-Lipo-siRNA, which indicated that the sterical hindrance effects of long-chain PEG molecules did play a key role in protecting liposomes from the interaction with serum proteins instead of shielding of positive charge. These results suggested that PEG- or RGD-modified liposomes would have a longer circulation time in vivo than that of unmodified liposomes.

Interestingly, from the results of in vitro and in vivo, the optimal cellular transfection and tumor inhibition were found in the group that treated with the targeted 1% RGD-Lipo-siRNA formulation when compared to those treated with other formulations that indicated the 1% molar ratio of RGD modification (equivalent to 2% mol ratio of PEG-DSPE) on the surface of lipoplex might be the best one for targeting the integrin receptors overexpressed on tumor neovasculature endothelial cells and tumor endothelial cells. As shown in the current data (Figs. 1–3), the cellular transfection effect resulted from 2% PEG-Lipo-siRNA was very close to that of 1%

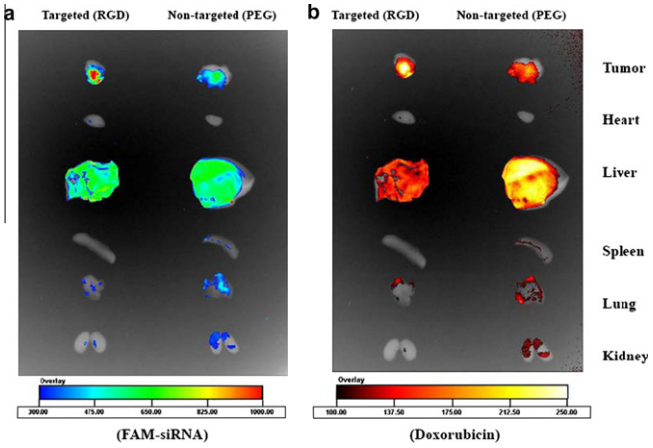

Fig. 7. Typical ex vivo imaging of tumor and organs excised from balb/c nude mice xenografted drug-resistant MCF7/A tumor at 4-h post-injection of siRNA-lipoplex and liposomal DOX. (a) FAM-siRNA: λ_{ex}: 490 nm, λ_{em}: 540 nm; (b) Doxrubicin: λ_{ex}: 480 nm, λ_{em}: 590 nm. Left of each figure stands for targeted (RGD) and right stands for non-targeted (PEG). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RGD-Lipo-siRNA, but more than those of 2% RGD-Lipo-siRNA (equivalent to 4% mol ratio of PEG-DSPE) and 4% PEG-Lipo-siRNA. These results suggested that similar molar ratio of DSPE-PEG in the composition of lipoplexes would have the similar effects on cellular transfection, but higher molar ratio of DSPE-PEG in lipoplex would attenuate transfection effect. This study showed that PEGylated liposomes (PEG- or RGD-modified) could significantly enhance the cellular uptake of siRNA when compared to the unmodified liposomes (Fig. 1), which might be due to the increased liposome fluidity and the enhanced internalization effect. Meanwhile, the lower P-gp gene silencing effect was found in the formulation with the higher molar ratio of PEG modification (2% mol RGD- or 4% mol PEG-modified lipoplexes) when compared to that of 1% RGD- or 2% PEG-modified lipoplexes (seen from in vitro and in vivo data), which might be attributed to the cellular internalization affected by the stronger sterical hindrance effect of long-chain PEG (equivalent to 4% mol ratio of PEG-DSPE in liposomes) and the uneasy siRNA escape from endosomes that involving interaction between lamellar phase stabilization of PEGylated lipids and endosomal membranes [35–37]. The cellular uptake of liposomal drugs, in general, is mainly influenced by liposome fluidity and sterical hindrance effect; so the internalization effect might be dependent on the molar ratio of DSPE-PEG in liposomes. But for siRNA delivery, besides internalization effects, the endosomal release of siRNA in plasma would play a key role in the following gene silence of RNAi. Therefore, we believe that there must exist an optimal ratio of PEG- or RGD- modification in siRNA-lipoplex delivery systems.

For in vivo delivery of siRNA, the small size (~150 nm) of lipoplexes ensured an enhancement in permeation and retention effect in solid tumors because the vasculature and endothelial junctions in these tissues become leaky (pore size ~400 nm) [38]. Besides, the positive charge of siRNA-lipoplexes also contributed to an increased uptake by the endothelial cells of blood vessels in tumor tissues [39]. These reasons could explain why non-targeted lipoplexes also exhibit tumor inhibition effect to some extent.

However, despite the co-delivery or sequential treatment of cytotoxicity drug and siRNA used for drug-resistant tumor therapy had been reported [14,17], the sequential treatment strategy for drug-resistant tumors with RGD-modified liposomes containing P-gp targeted siRNA or DOX still remains unexploited. In the present study, we used RGD-modified cationic liposomes for targeted delivery of siRNA and RGD-modified neutral liposomes for targeted delivery of DOX into cancer cells. The results of the present study showed that sequential administration of RGD-Lipo-siRNA(MDR1) and RGD-SSL-DOX could accumulate in the same tumor tissues with integrin receptors-mediated pattern. From the data of intracellular distribution of siRNA and DOX (Fig. 4), it was shown that there was no interaction or no interference happened between these two drugs after sequential treatment of RGD-Lipo-siRNA(MDR1) and RGD-SSL-DOX. Also, enhanced intracellular accumulation of DOX was observed with the treatment of RGD-Lipo-siRNA(MDR1) (Fig. 2). Furthermore, as well, enhanced tumor targeting of in vivo siRNA delivery was seen in the RGD-modified lipoplex when compared to that of other non-targeted (only PEG-modified) lipoplexes (Figs. 6 and 7). Subsequently, the best in vivo tumor therapeutic efficacy was observed in the animals treated with the targeted RGD-modified dual agent delivery systems (Fig. 5). Therefore, we are able to verify our hypothesis that tumor growth inhibition of DOX followed by down-regulation of P-gp by siRNA is required for effective therapy for multi-drug resistance tumors.

The best tumor therapy was obtained through sequential treatment of P-gp targeting siRNA and DOX based on RGD-modified liposomes. However, drug-resistant tumor growth was still seemed to be not inhibited completely, which might be resulted from the following reasons: First, the level of tumor inhibition is dependent on the dosage regimen and dose rate of DOX or siRNA, suggested that the optimal administration approach should be further exploited. A second possibility is the complicated mechanisms of multi-drug resistance, such as P-glycoprotein efflux and Bcl-2 anti-apoptotic activity [14], thus will result in incomplete therapeutic effect of multi-drug-resistant cancer.

In conclusion, different RGD-modified delivery systems for siRNA and DOX were developed separately for the stability of liposome formulations and the convenience of dosage fractionation, and the optimal therapeutic efficacy was obtained in the mice xenografted MCF7/A drug-resistant tumor with the sequential treatment of these two targeted liposome formulations. In future studies, the pharmacokinetics of both siRNA and DOX should be further investigated. Additionally, a more complicated system that involves more drug-resistant mechanisms should be further studied for completely reversing multi-drug resistance.

Acknowledgements

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