Supplementary material

Biodistribution of TAT or QLPVM coupled to receptor targeted liposomes for delivery of anticancer therapeutics to brain *in vitro* and *in vivo*

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Methods

Materials

1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP), 1,2-dioleoyl-snglycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DOPE-lissamine rhodamine), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were procured from Avanti Polar Limited (Birmingham, Alabama). 3-(N-succinimidyloxyglutaryl) aminopropyl, polyethyleneglycol-carbamyl distearoylphosphatidyl-ethanolamine (DSPE-$PEG_{(2000)}$-NHS) was procured from Biochempeg Scientific Inc. (Watertown, Massachusetts). Doxorubicin HCl was purchased from MedChem Express (Monmouth Junction, New Jersey). Erlotinib was bought from Cambridge Chemicals (Woburn, Massachusetts). Transferrin (Tf), Cholesterol (Chol) and Chitosan (50 kDa) were obtained from Sigma-Aldrich Company (St. Louis, Missouri). HIV-1 TAT (TAT) and QLPVM were obtained from Zhejiang Ontores Biotechnologies Co., Ltd (Zhejiang, China). Fetal bovine serum (FBS) was procured from Omega scientific Inc. (Tarzana, California). Dulbecco's modified eagle medium (DMEM) and Dulbecco's phosphate buffered saline (DPBS) were purchased from Mediatech Inc. (Manassas, Virginia). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar (Ward Hill, Massachusetts). Polyethylene terephthalate (PET) thincerts, cell culture inserts were bought from Greiner Bio-One

International (Monroe, North Carolina). Poly (D, L-lactide-co-glycolide) 50:50 was purchased from Polyscitech (West Lafayette, Indiana). All the chemicals used were of analytical grade. Glioblastoma (U87) and Brain endothelial cells (bEnd.3) were procured from American Type Culture Collection (ATCC, Rockville, Maryland).

Synthesis of DSPE-PEG$_{(2000)}$-CPPs and DSPE-PEG$_{(2000)}$-Tf

Synthesis of DSPE-PEG$_{(2000)}$-CPPs: The CPP was coupled to the distal end of the activated DSPE-PEG$_{(2000)}$-NHS through nucleophilic substitution reaction with some modification as per previously published reports [1,2]. Briefly, DSPE-PEG$_{(2000)}$-NHS and CPP were dissolved in an anhydrous dimethylformamide (DMF) at a molar ratio of 3:1 followed by pH adjustment between 8-9 using triethylamine. The reactants were stirred for 3 days at room temperature. Further, reactant mixture was placed in a dialysis bag MWCO 3.5 KDa for 48 h to separate out the free uncouple CPP from the resulting coupled product. The final product was freeze-dried and stored at -20^{o} C until used. Micro bicinchoninic acid (BCA) assay and HPLC were used to confirm the coupling of TAT and QLPVM, respectively.

Synthesis of DSPE-PEG$_{(2000)}$-Tf: The coupling of Tf was performed using nucleophilic substitution reaction to the terminal end of DSPE-PEG$_{(2000)}$-NHS, as previously reported [2–4]. Briefly, DSPE-PEG$_{(2000)}$-NHS along with Tf (125 µg /µ mole of lipid) were dissolved in an anhydrous DMF. The pH of reactant mixture was adjusted to 8-9 using triethylamine. With the help of magnetic stirrer the reactant mixture was stirred for 24 h at room temperature. The uncoupled Tf was separated out by passing the reaction mixture to G-100 Sephedex column. Using micro BCA assay, the coupling efficiency was determined, as per previously published article [2]. Tf and DSPE-PEG$_{(2000)}$-NHS were used as standard and control, respectively for the studies.

Preparation of dual functionalized liposomes and drug loading

CPP-liposomes were prepared using thin film hydration method while post-insertion method was used to formulate dual-functionalized liposomes, as per previously published reports [2,4,5]. Briefly, DSPE-$PEG_{(2000)}$-CPP along with erlotinib and other phospholipids were dissolved in chloroform: methanol (2:1, v/v) solvent mixture at a molar ratio of 45:45:2:4 (mole %). With the help of rotavapor (Buchi Rotavapor RII, New Castle, DE), the solvent mixture was evaporated to form a dried lipid film. CPP-liposomes were formed by hydrating the lipid film with 300 mM citric acid buffer pH 5.0. Then, Tf-CPP liposomes were prepared by adding DSPE-$PEG_{(2000)}$-Tf micelles to preformed CPP-liposomes and stirred overnight on magnetic stirrer at room temperature. Doxorubicin was loaded into liposomes using pH gradient method. The pH gradient was created by titrating the external pH of liposomes with 300 mM sodium carbonate followed by addition of doxorubicin and incubating for 1 h at 50 °C. Then, the liposomes were pass through G-100 sephadex column to separate unentrapped Dox and Erlo.

The entrapment efficiency of doxorubicin and erlotinib was quantified as per published reports [2,4,6]. Briefly, before and after passing through the column the liposomes were lysed using methanol and triton x-100 (0.5% v/v), followed by centrifugation at 3000 rpm for 10 min. Then, the supernatant liposomal lysate was injected into high performance liquid chromatography (HPLC) to determine the entrapment efficiency. With some modifications in the method, the analysis of doxorubicin was performed using C-18 column (Thermoscientific Hypersil Gold, 5 µm, 250 x 4.6 mm) at a wavelength 234 nm [2,6]. The mobile phase comprised of 0.2 M phosphate buffer, pH 5.5: acetonitrile (70:30) with a flow rate of 1 ml/min at room temperature. The analysis of erlotinib was done using C-8 column (Thermoscientific Hypersil BDS, 5 µm, 250

x 4.6 mm) at a wavelength of 246 nm. The mobile phase consisting of 0.2 M potassium phosphate, pH 3.0: acetonitrile (50:50) with a flow rate of 0.750 ml/min at room temperature [2,6].

Lissamine-rhodamine labeled liposomes was prepared by dissolving 0.5 mole% of lissamine-rhodamine coupled DOPE as liposomes membrane marker along with other prior to formation of thin lipid film.

Characterization of liposomal nanoparticles

The liposomal formulations were appropriately diluted with phosphate buffer saline (PBS) before determining the particle size and zeta potential using zetasizer ZS 90 (Malvern Instruments, Worcestershire, UK) at 25^{o}C. The instrument was equipped with 5 mW He-Ne laser of wavelength 633 nm. The sample filled cuvettes were placed in the path of laser and the data was collected at a scattering angle of 90^{o}.

In vitro cytotoxicity

The in vitro biocompatibility of liposomes was determined by MTT assay. U87, bEnd.3 and glial cells lines were used to study the cytotoxic potential of liposomes. The assay was performed as per previously published reports [2,5]. Briefly, cells were seeded at a density of 1,000 cells in each well of 96 well plates in 200 µl of DMEM supplemented with 10% FBS and 1% pen-strep and incubated at 37^{o}C under 5% CO_2 atmosphere. After 24 h, cells were incubated for 2 h with different phospholipid concentrations of liposomes in serum free media. Subsequently, the formulation was replaced with fresh serum containing media and cells were further incubated at 37^{o}C under 5% CO_2 atmosphere for 48 h. After 48 h, the cytotoxic potential of liposomal formulations was evaluated by determining the viability of cells using MTT assay. Untreated cells were considered as control group under the same cell culture conditions.

Cellular uptake assessment

The cellular uptake assessment of liposomes was performed in U87, bEnd.3 and glial cell lines. Briefly, 6 X 10^5 cells were seeded in each well of 6 well plates and incubated at 37^{o}C under 5% CO_2 atmosphere. Cells were incubated for 24 h prior treatment. Cells were incubated for 2 h with 200 nMoles of phospholipid concentration of different doxorubicin and erlotinib loaded liposomal formulations. After treatment, the formulation was removed and cells were washed with DPBS, pH 7.4. To determine qualitative uptake, the cells were fixed using cold methanol followed by staining the nucleus of cells with 1 ml of Hoechst 33342 (1 µg/mL). Then, the cells were observed under Leica DMi8 fluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL). The quantitative estimation of Dox and Erlo uptake was performed by lysing the cells using triton x-100 (0.5% v/v), followed by the extraction of drugs in methanol from cell lysate. Then, using centrifuge, the drug extract cell lysate was centrifuged at 10,000 rpm at 4^{o}C for 10 min. Subsequently, the supernatant was injected into HPLC to determine percent drug uptake in cells. The analysis was performed as per the above mentioned method for the quantification of Dox and Erlo entrapment efficiency [2].

Hemolysis study

Cell penetrating peptides have been reported to be cytotoxic for erythrocytes due to their cationic charge [5,7]. Hemolysis study was performed to check the in vitro biocompatibility of liposomal formulations prior to in vivo administration. The intravenous injection of these liposomes may interact with negatively charge erythrocytes' membrane and cause lysis of erythrocytes. Briefly, rat blood was collected in an EDTA containing tube, followed by washing with sterile PBS, pH 7.4. Then, the tube was centrifuged at 2000 rpm for 10 min to separate erythrocytes. Different phospholipid concentration of liposomes were incubated with predetermined number of

erythrocytes (1.5 x 10^7) at 37ºC. After 60 min of incubation, the samples were centrifuged at 2000 rpm for 10 min. Using spectrophotometer (SpectraMax® M5, Molecular devices, Sunnyvale, CA), the absorbance of supernatant was noted at 540 nm. The samples treated with PBS and triton x-100 demonstrated 0% and 100% hemolysis, respectively were considered as controls. The percent hemolysis was quantified as per previously published reports [2,5].

In vitro co-culture endothelial barrier

The *in vitro* co-culture endothelial barrier was prepared, as per the previously published reports [2,5,8]. Briefly, bEnd.3 cells (37,500 cells/cm^2) and glial cells (15,000 cells/cm^2) were seeding on luminal side and abluminal side of the insert, respectively. Then, the insert was further cultured for 6 days to form a tight barrier. The confluency of the barrier was checked each day and the culture medium of the barrier was changed in every 2 days. The integrity of both co-culture endothelial (bEnd.3 and primary glial cells) and monolayer (bEnd.3 cells) barrier models was determined by measuring the transendothelial electrical resistance (TEER) using EVOM2 with STX2 (World Precision Instruments, Sarasota, FL).

3-dimensional tumor growth inside PLGA-chitosan scaffold

The porous PLGA-chitosan scaffold was prepared by emulsion freeze dry method, as per previously published reports [2,4]. Briefly, chitosan (50 mg/ml) and polyvinyl alcohol (PVA; 15 mg/ml) were dissolved in 10 ml of acetic acid buffer, pH 4.5. Separately, 1 g of poly (D, L-lactide-co-glycolide) (50:50) (PLGA) was dissolved in 5 ml of dichloromethane. With stirring slowly, the PLGA solution was mixed to the chitosan-PVA mixture at a rate of 2ml/min to form an emulsified paste. To this paste, 500 µl of 0.1 % w/v of collagen solution was added, followed by pouring this paste into a rod-shaped mold to freeze dry. After freeze drying, the rod shape scaffold was cut into

2 mm circular disc shape and further treated with 5M NaOH solution and washed with three times with sterile PBS, 7.4 to remove excess of NaOH. The scaffolds were soaked in 70% ethanol and then in DMEM with 30% FBS overnight. Next day, the scaffold was seeded with 5 x 10^5 U87 cells on the surface and incubated for 6 h. After that, 30% FBS containing DMEM was added to the scaffold and media was replaced in every 2 days. Hematoxylin and eosin (H&E) staining of scaffold was performed at predetermined time intervals to evaluate the growth of tumor.

Transport across the endothelial barrier

The transport of liposomes was determined across the endothelial barrier using *in vitro* brain tumor model, as per previous reports [2,5]. The *in vitro* brain tumor model was prepared by placing the co-culture endothelial barrier (containing bEnd.3 and glial cells) on U87 tumor grown scaffold on day 14 and further cultured for addition 7 more days. On day 21, the upper compartment of the *in vitro* brain tumor model was treated with different liposomal formulations (200 nMoles) in 500 µl of DPBS with 10% FBS. The samples were collected at predetermined intervals. In addition, the tumor cells in the scaffold were lysed to extract the drug as per described in the cellular uptake procedure. The sample was then centrifuged at 4 ^oC for 10 min at 10,000 rpm. The liposomal transport was analyzed using HPLC as described in the cellular uptake.

In vitro anti-tumor efficacy of liposomes using in vitro brain tumor model

The *in vitro* brain tumor model was used to study anti-tumor efficacy of liposomes, as per previously published reports [2,5]. Concisely, the media in the upper compartment of the model was replaced with different Dox and Erlo loaded different liposomal formulations (200 nMoles) in 500 µl of DPBS with 10% FBS. After 24 h treatment, the scaffold was again incubated with DMEM with 30% FBS for additional 6 more days at 37^oC under 5% CO_2 atmosphere before

determining the percent tumor viability using MTT assay. The media was changed in every two days. With the help fluorescence imaging, the anti-tumor efficacy was further confirmed. After study, the treated scaffolds were stained with live/dead cell staining (Biotum Inc., Fremont, CA), as per the manufacturer's protocol. After staining, the treated scaffolds were snap frozen in OCT and with the help of cryostat 20 µm thick sections were cut. The slides were observed under fluorescence microscope (DMi8 fluorescence microscope Leica Microsystems Inc., Buffalo Grove, IL).

Data analysis

Analysis of data was done using Graphpad Prime 5.0 for windows (GraphPad Software, Inc., La Jolla, CA). Statistical analysis between groups were performed by either Student's t test, one or two way ANOVA. A $p < 0.05$ was considered statistically significant. All the quantitative data was represented as a mean ± standard deviation (SD).

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