Supplementary Information

Aptide-conjugated liposome targeting tumor-associated fibronectin for glioma therapy

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Materials and Methods

Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (POPG), polyethylene glycol($_{2000}$)-DSPE (ammonium salt) (PEG$_{2000}$-DSPE), N-maleimide-PEG$_{2000}$-DSPE (ammonium salt) (Mal-PEG$_{2000}$-DSPE), plant cholesterol (Chol), and a mini handheld extruder set were purchased from Avanti Polar Lipids (Alabaster, USA). An aptide specific for the EDB domain of fibronectin (APT$_{EDB}$) with an additional cysteine in the β-hairpin constant loop region, *N'*-CSSPIQGSWTWENGK(**C**)WTWGIIRLEQ-*C'*, was custom-synthesized by Anygen Corp (Jangsung, JeonNam, South Korea). The hydrophilic anti-cancer drug, doxorubicin hydrochloride (Dox), was purchased from Boryung Pharmaceuticals Co. Ltd (Seoul, South Korea). Sepharose CL-4B column was purchased from Sigma-Aldrich (Missouri, USA). Mounting solution was purchased from Dako (Glostrup, Denmark). All other chemicals were of reagent grade and were used as received. All animal experiments were done according to the rules and regulations of animal care and handling procedures of Gwangju Institute of Science and Technology (GIST).

Synthesis of APT_{EDB}-PEG_{2000}-DSPE

APT_{EDB} with an additional cysteine residue was dissolved in DMSO and Mal-PEG_{2000}-DSPE was dissolved in chloroform. The conjugation reaction was carried out at an APT_{EDB}: Mal-PEG_{2000}-DSPE molar ratio of 1:2 under inert conditions for 12 h at ambient temperature. The conjugation efficiency was determined by MALDI-TOF analysis, which was carried out by Anygen Corp. The conjugation was also determined by a thin-layer chromatography (TLC) method using chloroform:methanol:water at a molar ratio of 74:36:6 (Fig. S1b).[1]

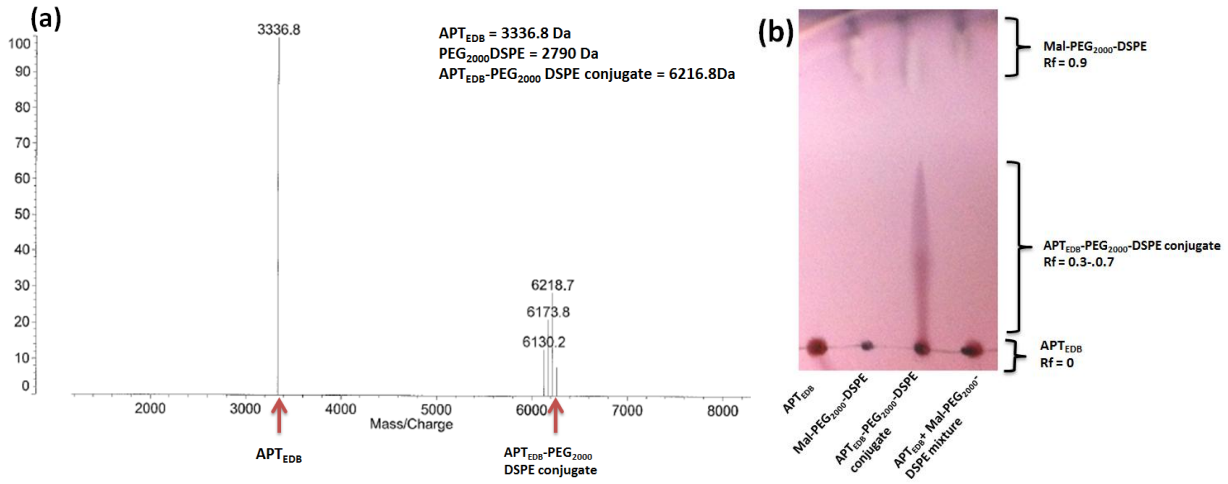

Fig. S1 The targeting ligand APT_{EDB} was conjugated to Mal-PEG_{2000}-DSPE via thiol-maleimide chemistry. The molecular weight (MW) of APT_{EDB}-PEG_{2000}-DSPE was determined by MALDI-TOF analysis to be approximately 6200 Da, consistent with the theoretical MW, confirming the success of the conjugation reaction. The conjugate is seen clearly in TLC plates, as indicated by a broad smearing. The peptide Rf value is 0 whereas the Rf value of Mal-PEG_{2000}-DSPE was found to be 0.9. A mixture of these two components resulted in the appearance of distinct dots rather than a smear, confirming that only the peptide-lipid conjugate is detected in the Rf range 0.3-0.7.

Preparation of liposomes

An anionic lipid film of POPC:Chol:POPG (molar ratio, 4:3:3; +/N/- charge ratio, 6:1:6) was prepared as described previously[2]. Briefly, all components were added into a glass vial and dried under a vacuum and further lyophilized overnight to remove all remaining chloroform. During rehydration, 1 ml of HEPES-buffered 5% glucose (HBG) was added to the lipid film to yield a

liposome solution with a final concentration of 2 mg/ml. After brief sonication, the solution was extruded through a 100-nm polycarbonate membrane using a mini handheld extruder. At least ten extrusions were done for each sample to ensure the formation of uniform-sized liposome. Liposomes incorporating APT_{EDB}-PEG_{2000}-DSPE were added to the original liposome formulation at a concentration of 2.5 wt%; liposomes without APT_{EDB}-PEG_{2000}-DSPE served as a control. For Dox loading, doxorubicin hydrochloride was dissolved in HBG buffer at a final concentration of 50 μg/ml of liposome. Dox-loaded liposomes were then purified by size-exclusion chromatography (CL-4B column). A schematic depiction of liposome preparation and transmission electron microscopy (TEM) images of liposomes after extrusion are illustrated in Fig. S2.

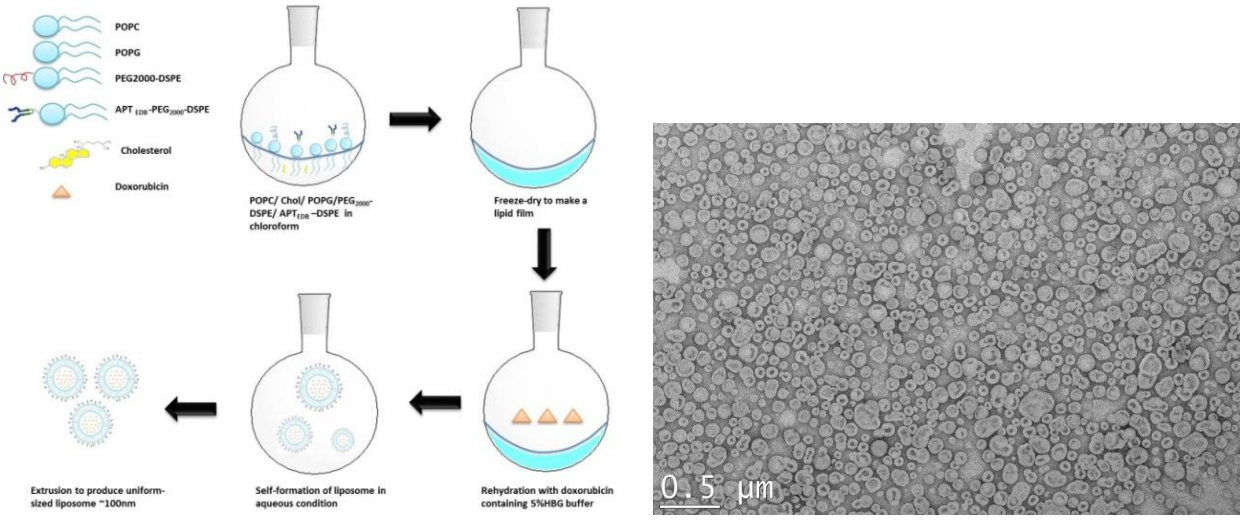

Fig. S2 Schematic representation of liposome formation. All components were dissolved in chloroform to form a lipid film. During rehydration, Dox was included in the HBG5% buffer. Liposomes form automatically under aqueous condition. After CL-4B column purification to remove free Dox, liposomes were extruded through a 100-nm polycarbonate membrane. *Right:* TEM image of liposomes after extrusion.

Size distribution and zeta potential

The particle size and zeta potential of LS and APT_{EDB}-LS were determined by dynamic light scattering (DLS) analysis using an ELS 8000 electrophoretic light-scattering apparatus (Otsuka

Electronics, Osaka, Japan) at ambient temperature. The data for each sample were obtained from three replicates (Table S1).

Table S1 The size and zeta potential of LS and APT_{EDB}-LS before and after encapsulating Dox.

Formulation	Average size (nm)	Average zeta potential
LS	**103.7 ± 15.3**	**-50.15 ± 1.51**
APT_{EDB}-LS	**118.0 ± 3.46**	**-39.40 ± 2.81**
LS (Dox)	**121.5 ± 16.39**	**-47.30 ± 2.23**
APT_{EDB}-LS (Dox)	**122.8 ± 10.75**	**-40.01 ± 1.11**

Drug encapsulation efficiency

After encapsulation of Dox, excess free Dox was removed by gel permeation chromatography using a CL-4B column. The column was pre-washed three times with HBS buffer and washed with 1 ml of 5% dextran to prevent non-specific binding of the formulation to the column. A total of fifteen 1-ml fractions were collected for each sample (LS and APT_{EDB}-LS). Liposome fractions were eluted in fractions 3-5 whereas free Dox were eluted in fraction 13-14. For Dox quantification in liposomes, the liposomes were first lysed by treating 500 μl of each collected fraction with 500 μl of ethanol:ethyl ether:isopropanol (1:2:1). The concentration of Dox was quantified fluorimetrically using an excitation wavelength of 488 nm and an emission wavelength of 595 nm. The encapsulation efficiency was then calculated as the drug loaded into liposomes divided by the total drug used.

Cell culture

The PC3 human prostate cancer cell line, GL26 mouse glioma cell line and NIH 3T3 human fibroblast cell line were used. All cells were maintained at 37°C in a humidified 5% CO_2 environment. The GL26 mouse glioma cell line was kindly provided by Prof. Yong-kil Hong

(Department of Neurosurgery, College of Medicine, Catholic University, South Korea) and was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The PC3 human prostate cancer cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The NIH3T3 cell line was obtained from Korea Cell Line Bank (KCLB) and was maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Examination of the level of fibronectin EDB expression in various cell lines

Expression of the EDB domain of fibronectin in various human cancer cell lines was determined by conventional RT-PCR and real-time qRT-PCR. Cells were first collected and RNA was isolated using RiboEx with a GeneAll RNA isolation kit. Synthesis of cDNAs was carried out using 1 µg of total RNA from each sample. The following primers were used for detecting the common region of fibronectin mRNA: 5'-TAG CCC TGT CCA GGA GTT CA-3' (5'-primer) and 5'-CTG CAA GCC TTC AAT AGT CA-3' (3'-primer), yielding a 346-bp fragment. The primers for detecting only the EDB domain of fibronectin were as follows: 5'-AAC TCA CTG ACC TAA GCT TT-3' (5'-primer) and 5'-CGT TTG TTG TGT CAG TGT AG-3' (3-primer), yielding a 263-bp fragment. PCR reactions were carried out using a BioRad thermocycler. The PCR protocol consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 2 min (extension), and a final extension at 72°C for 7 min.[3] PCR products were analyzed by agarose gel electrophoresis. For real-time RT-PCR, 1 µg of cDNA was added to 4 µl of ultra-pure water and 5 µl of SYBR Green real-time mixture (Takara, Tokyo, Japan).

Cell binding of liposomes *in vitro*

Cellular binding and internalization of both LS and APT_{EDB}-LS were studied using fluorescence detection. LS and APT_{EDB}-LS were fluorescently labeled with rhodamine-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine at 0.1 wt%. GL26 glioma cells, PC3 human prostate cancer cells and NIH 3T3 human fibroblast cells were grown in 10% RPMI-1640 supplemented with FBS to ~80% confluence on coverslips (12 × 12 mm; Fisher Scientific, Texas, USA) and the medium was replaced with serum-free medium containing 200 µg/ml of fluorescently labeled LS or APT_{EDB}-LS. After a 1-hr incubation at 37°C, cells were washed three times with PBS and fixed with 4% (w/v) paraformaldehyde (PFA). Coverslips with fixed cells were mounted on slide glass with Dako® mounting media and examined using an Olympus Fluoview 1000 confocal microscope (Olympus Imaging Co., Tokyo, Japan).

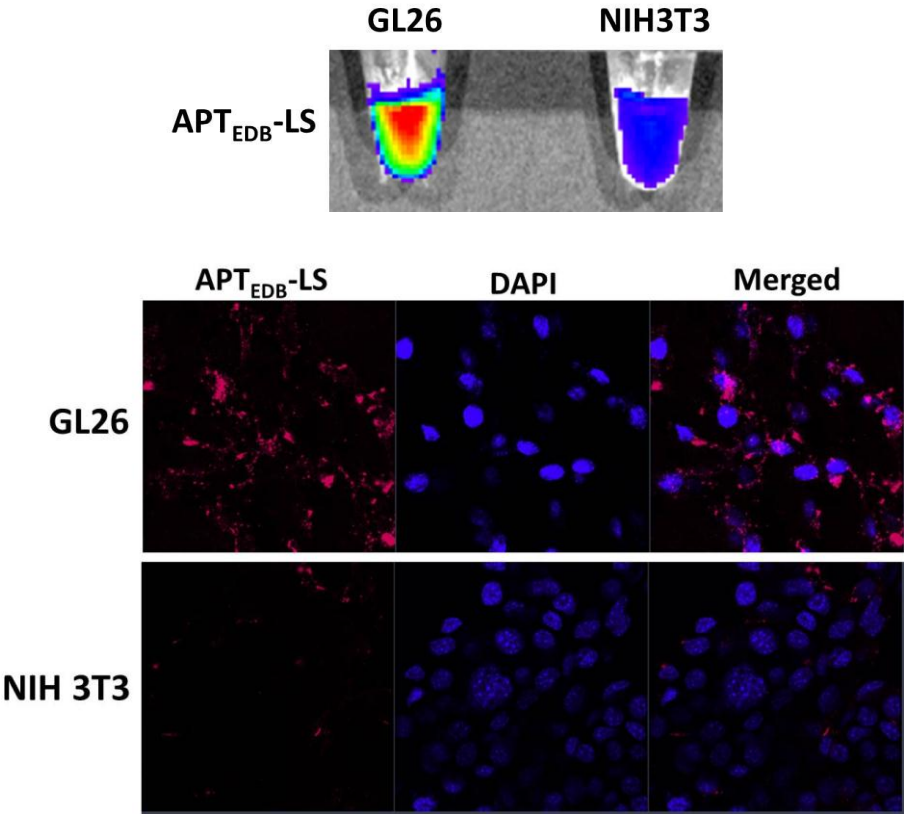

Fig. S3 The uptake of LS and APT_{EDB}-LS in EDB positive GL26 cell line and NIH3T3, a normal human fibroblast cell line. (a) The uptake of APT_{EDB}-LS in GL26 and NIH3T3 ($1x10^5$ cell population) observed by IVIS imaging. (b) Uptake of APT_{EDB}-LS in GL26 and NIH3T3 as evidenced by confocal microscopy.

In vitro competition assay

PC3 and GL26 cells were grown on glass coverslips until reaching ~80% confluence. Cells were pre-treated with different concentrations (100 μg/ml and 500 μg/ml) of free APT_{EDB} peptide for 30 mins in serum-free media. Then, rhodamine-labeled APT_{EDB}-LS was added and cells were incubated for an additional 30 min. Thereafter, cells were washed with PBS, fixed with 4% PFA, and mounted on microscope slides for viewing under confocal microscope.

Assessment of targeting ability of APT_{EDB}-LS(Dox) in various cancer cells

PC3 and GL26 cells were grown on glass coverslips until reaching ~80% confluence. Each cell line was treated with APT_{EDB}-LS(Dox) (40 μg Dox/ml) for 1 h at 37°C in serum-free media. The uptake efficiency was compared to those of Dox and LS(Dox) groups.

In vivo imaging with liposomes

Balb/C nude mice were used for mouse allograft models. The GL26 allograft model was created by inoculating 1×10^7 cells into the right flank of the mouse. After 2 weeks, when tumors had grown to about 100 mm^3, mice (3 mice/group) were injected via the tail vein with 200 μl of LS or APT_{EDB}-LS for subsequent imaging. Six hours after receiving injections, mice were sacrificed and their organs were excised. Tumors and other organs were examined *ex vivo* by IVIS imaging (Perkin Elmer, Massachusetts, US).

In vivo anti-tumor efficacy

Anti-tumor efficacy was studied using the GL26-cell-bearing flank-tumor model, as described above. After tumor volumes had reached 50-80 mm^3, mice were randomly assigned to four groups (n=7) as follows: group 1, saline; group 2, free Dox; group 3, LS(Dox); and group 4, APT_{EDB}–LS(Dox). All treatments were administered by intravenous injection of four doses of

Dox (4 mg/kg) delivered every other day. Tumor volumes were measured every 2 days until the end of experiment. The tumor weights for each group are illustrated in Fig. S3.

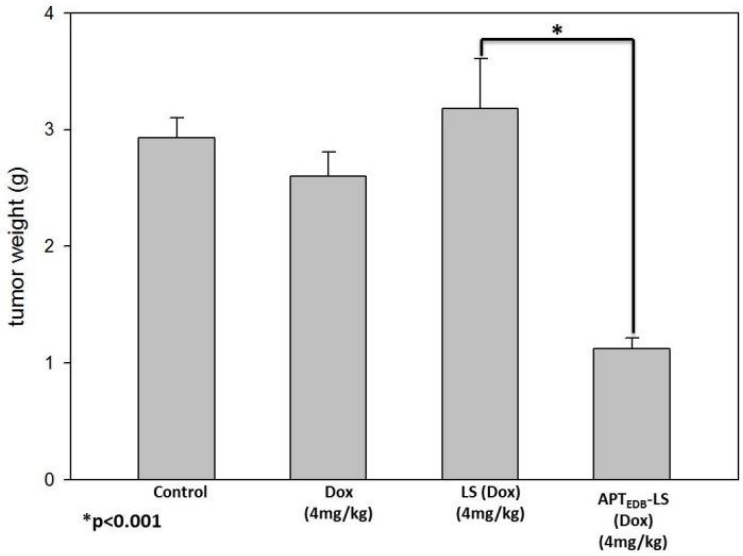

Fig. S4 Tumor weights in each group after *in vivo* anti-tumor therapy in the GL26 allograft model.

References

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3. S. Matsui, T. Takahashi, Y. Oyanagi, S. Takahashi, S. Boku, K. Takahashi, K. Furukawa, F. Arai and H. Asakura, *J Hepatol*, 1997, **27**, 843-853.