Title: Single Protein Encapsulated Doxorubicin as an Efficacious Anticancer Therapeutic

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MATERIAL and METHODS

Material and instruments

HSA (25% solution) and doxorubicin hydrochloride were purchased from Octapharma USA and Synbias Pharm, respectively. Methanol, ethanol, Spectrum dialysis membranes (12-14 kD MWCO), and Zeba desalting spin columns were from VWR. Doxorubicin UV spectrum measurement and quantitation were conducted on a UV-1600 PC spectrometer (VWR). A FRET master fluorometer (Photon Technology International) was used to measure fluorescence. Particle size distributions were determined by a Zetasizer Nano dynamic light scattering system (Malvern Panalytical). DOX-HSA mixtures were prepared by adding the desired amount of aqueous DOX solution and HSA solution into PBS buffer to form DOX-HAS mixtures with the desired ratios of DOX to HSA.

Preparation of SPEDOX complexes and their analysis

DOX quantitation was based on its strong absorbance between 450-550 nm. Linear standard calibration curves were used to calculate DOX's molar extinction coefficients ($\boldsymbol{\varepsilon}_{nm}$) under different conditions – $\boldsymbol{\varepsilon}_{481}$ = 11,500 cm^{-1} M^{-1} and $\boldsymbol{\varepsilon}_{547}$ = 3,900 cm^{-1} M^{-1} in PBS buffer, $\boldsymbol{\varepsilon}_{481}$ = 11,800 cm^{-1} M^{-1} in acetate buffer. For SPEDOX in PBS, $\boldsymbol{\varepsilon}_{547}$ = 3,900 cm^{-1} M^{-1}.

All SPEDOX complexes were prepared according to the following general procedure described briefly. To a 50 mL round flask equipped with a magnetic stir bar, were added 4.0 mL of 25 % HSA solution (1.0 g of HSA), 2.0 mL of deionized water, 1.0 mL of 50% methanol-water

mixture, and 1.0 mL of 56% ethanol-water mixture. The resulting mixture was stirred for 5 minutes at room temperature. Separately, 27 mg to 80 mg of doxorubicin hydrochloride (the pre-determined DOX/HSA ratios) was mixed with 4.0 mL of deionized water in a 15 mL centrifuge tube. Different amounts of DOX were used to produce SPEDOX complexes containing different [DOX]/[HSA] ratios ranging from 3 to 9. The mixture was vortexed thoroughly until all solid was dissolved to form a red solution, which was then added into the above HSA solution while stirring, followed by adding 1.0 mL of 56% ethanol-water mixture. Subsequently, the pH was brought up to 11.0 by using 1.0 M NaOH solution. The mixture was continuously stirred for specific times (from 7 to 12.6 hours) to achieve the desired binding strength between DOX and HSA. The encapsulation process was conveniently monitored by UV absorbance analysis of samples taken at different time intervals and diluted in PBS. The A_{547}/A_{481} ratio was then calculated and used as a reliable indicator for DOX encapsulation strength. Free DOX has maximum absorbance at 481 nm. The absorbance at the isosbestic point of 547 nm remained unchanged during the encapsulation process, thereby providing a constant reference point for ratio calculation. Once the desired binding strength was achieved, the mixture was concentrated by a high vacuum pump to remove most of the organic solvents. After adjusting the pH to 7.4, the resulting red solution was filtered through a 0.2 μm polyethersulfone membrane. The concentration of DOX in SPEDOX complexes was determined by UV absorbance in PBS using $\boldsymbol{\varepsilon}_{547}$ and [DOX]/[HSA] ratio was re-calculated. Finally, 20 mL of the solution containing a known amount of SPEDOX were aliquoted into 50 mL tubes and lyophilized to yield red powdered products. A series of SPEDOX complexes with different DOX loading (SPEDOX-1, SPEDOX-2, and SPEDOX-3 with DOX/HSA ratios = 4.0, 7.0, 9.0, respectively, and a constant A_{547}/A_{481} ratio = 0.54) and binding strength (SPEDOX-4, SPEDOX-5, SPEDOX-6 and

SPEDOX-7 with A_{547}/A_{481} ratios = 0.46, 0.49, 0.53, 0.56, respectively, and a constant DOX/HSA ratio = 9.0) were successfully prepared.

Fluorescence analysis was conducted with the excitation at 470 nm and emission spectra from 510-700 nm were recorded. For potassium iodide (KI) quenching experiments, different amounts of KI were added to s defined concentration of DOX, DOX-HSA mixture, or SPEDOX-6 to collect fluorescence spectra, F_{510} was used to calculate fluorescence quenching.

Dialysis

Membrane dialysis kinetics were examined to evaluate the relative DOX encapsulation strength and DOX release from SPEDOX complexes under two different pH conditions. A series of powdered samples containing 5.0 mg of DOX or DOX equivalent from SPEDOX-1 to SPEDOX-7 were each dissolved in 6.0 mL of PBS buffer (pH = 7.4) or acetate buffer (pH = 5.2) and the resulting clear red solutions were transferred into separate dialysis bags. Each dialysis bag was then placed into a jar containing 350 mL of PBS buffer that was previously degassed by nitrogen for 30 minutes. The jars were capped airtight and placed in a dark room to avoid UV radiation. At different time intervals, 2.0 mL of the buffer solutions in the jars were taken out and analyzed by UV spectrometry to determine the total amount of dialyzed-out free DOX, which was calculated using A_{481} and $\boldsymbol{\varepsilon}_{481}$. A separate series of dialysis studies were conducted in acetate buffer (pH 5.2) following the same procedure. Each dialysis study was conducted in triplicate.

In Vivo Study

All *in vivo* evaluations of SPEDOX complexes using mice were conducted at Charles River Laboratory in accordance with the approval of the Charles River Laboratory Institutional Animal Care and Use Committee.

PK studies in mice

Three female BALB/c mice per group were used for PK studies. A single dose of SPEDOX-6 at 12 mg/kg DOX equivalent was injected intravenously (IV) into each mouse. Blood samples were collected into EDTA-containing tubes at 5 min, 2 h, 4 h, and 24 h. Each sample was then equally split into two halves, resulting in two sets of blood samples. The samples from one set were passed through desalting spin columns to remove free DOX. Subsequently, both sets of the samples were treated with acidic acetonitrile to extract DOX, which was then quantitated by HPLC/MS analysis. The measured difference in DOX amount between the two sets of samples reflected the amount of DOX removed by desalting spin columns, and thus represented the amount of free DOX in blood samples.

Mouse heart tissue studies

Three female BALB/c mice per group and a single dose (IV) of SPEDOX-6 at 12 mg/kg DOX equivalent were used for this study. At 2 h and 24 h post IV injections, animals were sacrificed and heart tissues were harvested, weighed, and homogenized. Total DOX was extracted and quantitated by the same method as described above.

Evaluation of anti-tumor activity in vivo

MDA-MB-231 cell line-derived human breast tumor xenografts were produced in eight-

week old female athymic nude mice according to established methods. When tumor size reached 80-120 mm^3, designated as Day 1 of the treatment, mice were sorted into three treatment groups – vehicle, DOX, and SPEDOX-6 (n = 8 per group) . In the first antitumor efficacy study, the dosing schedule consisted of weekly IV injections for three cycles at a dose of 5 mg/kg free DOX, the maximum tolerated dose (MTD), or 10 mg/kg DOX equivalent SPEDOX-6 (2X of DOX's MTD). Both tumor volume and body weight were measured twice per week. The study endpoint was defined as a mean tumor volume of 2000 mm^3 in the vehicle group (saline only) or 20 days of treatment. Based on the results from this study, we conducted the second antitumor efficacy study, in which the doses of SPEDOX-6 were increased to 20 mg/kg and 30 mg/kg DOX equivalent (4X and 6X of DOX's MTD) with an endpoint of tumor volume reaching 2000 mm^3 or 29 days in the vehicle group (n = 4).

Statistical analyses

For *in vitro* studies, two-tailed statistical analyses were conducted. Prism summarizes test results as not significant (ns) at $P > 0.05$, significant "*" at $0.01 < P < 0.05$, "**" at $0.001 < P < 0.01$, and "***" at $P < 0.001$.

To evaluate the inhibitory effects of DOX (5 mg/kg) and SPEDOX-6 (10 mg/kg DOX equivalent) on MDA-MB-231 xenograft tumor growth, summary statistics of mean, standard deviation, median, and range were calculated for tumor volume changes and proportion of body weight changes over time from day 1 for the three groups (vehicle, DOX and SPEDOX-6) of eight mice. Data from day 1 were subtracted from values at a specific day for each mouse to calculate changes from day 1. These changes were divided by day 1 values to get the proportion changes. Same preprocessing was done in the study of the anti-tumor effects of 20 mg/kg and 30 mg/kg

DOX equivalent SPEDOX-6 in comparison with the vehicle treatment (four mice per group). Linear mixed model regression was done with tumor volume changes and proportion of body weight change as the outcomes in two models. Group and day effects were in the model with an interaction between group and day. A repeated effect for the animal was used to estimate correlation within mouse over time and an unstructured covariance structure was used because the variance was not constant over time. Post-hoc pair-wise comparisons for the primary outcome of tumor volume changes were made between groups at each time point at each specific day after day 1 with a Benjamini-Hochberg multiple comparisons procedure applied. A Tukey-Kramer adjustment was made for proportion of body weight changes for all pair-wise comparisons. A significance level of 0.05 was used for all tests. All statistical analyses were done using SAS 9.4 (SAS Institute, Cary, NC).

Figures and Tables

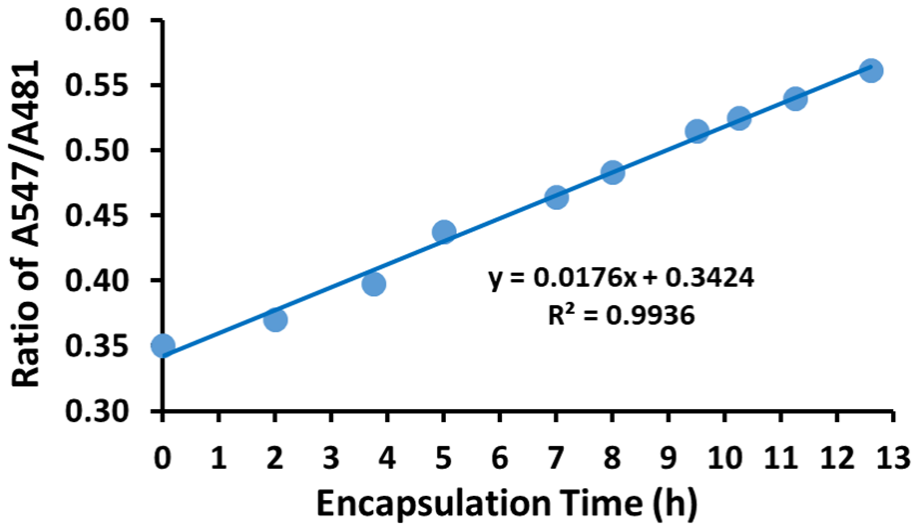

Figure S1: A linear relationship between A_{547}/A_{481} ratio change vs. encapsulation time.

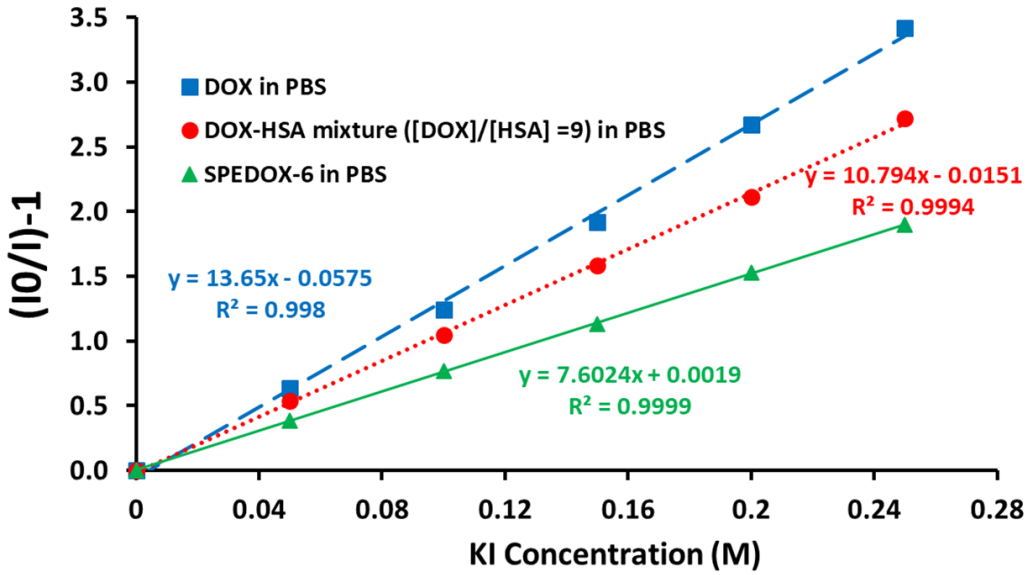

Figure S2: Stern-Volmer (S-V) plots of fluorescence quenching of DOX, DOX-HSA mixture, and SPEDOX by KI (potassium iodide): ■ - free DOX (170 nM), ● - DOX-HSA mixture ([DOX] = 130 nM + [HSA]= 15 nM), ▲ - SPEDOX-6 ([DOX]=500 nM and [HSA] = 56 nM).

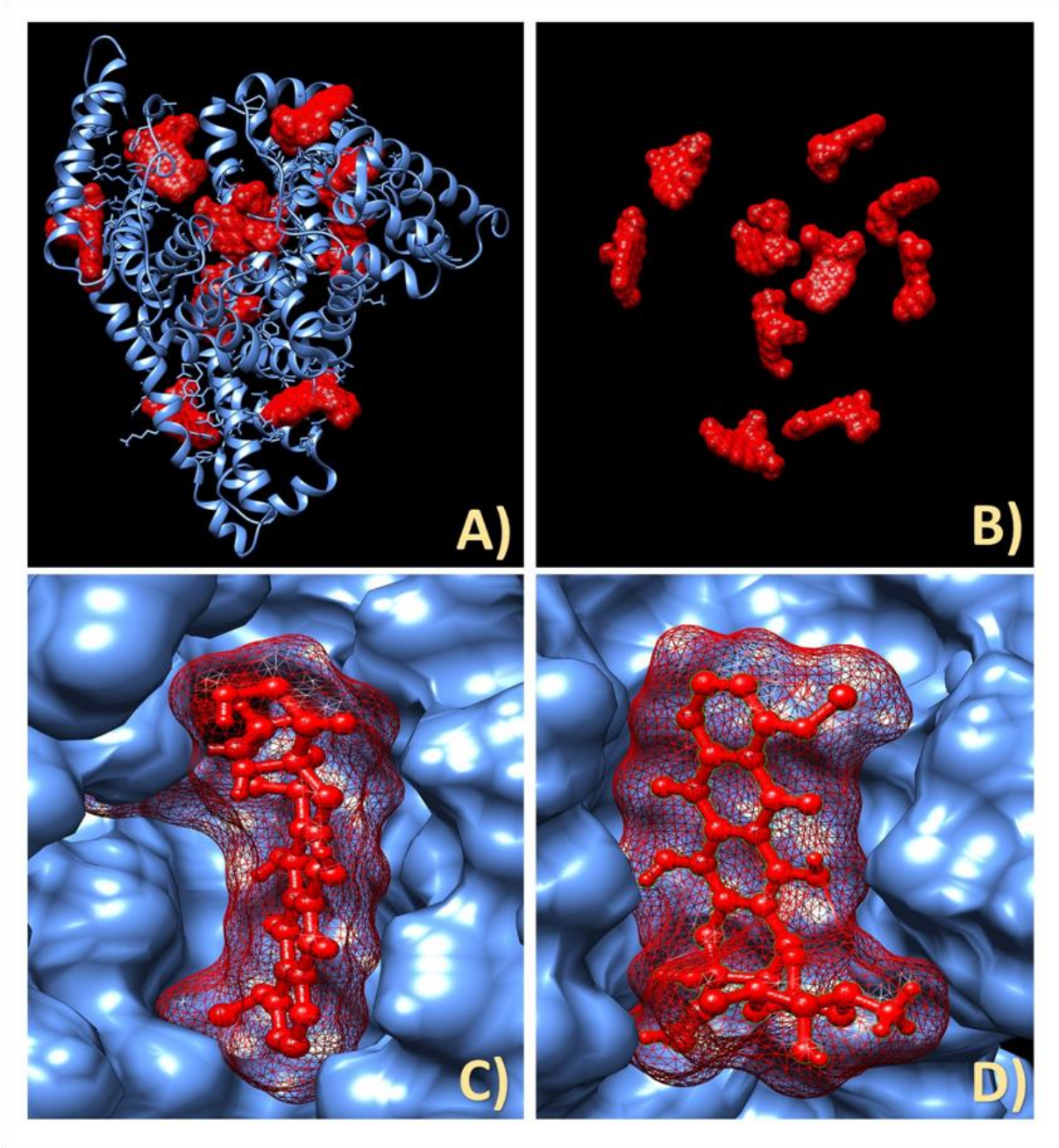

Figure S3: DOX docking onto HSA using UCSF Chimera (https://www.cgl.ucsf.edu/chimera/**) and AutoDock Vina (**http://vina.scripps.edu/**).** HSA and DOX structures were derived from PDB ID# 1E78 and 4ZVM, respectively. A) Top 10 DOX molecules docked at non-overlapping locations within HSA are shown. The DOX-HSA binding Gibbs free energy (ΔG^o) at the 10 docking sites was calculated to be -9.1, -8.7, -8.6, -8.0, -7.3, -7.2, -7.2, -7,1, -7.0, and -6.5 kcal/mol, corresponding to Kd values of 0.2, 0.5, 0.5, 1.0, 2.0, 5.0, 6.0, 7.0, 8.0 and 18.0 µM. The docking generated PDB file is supplied as a separate SI file named HSA-DOX complex.pdb. B) The relative locations of the 10 docked DOX molecules after HSA removal from the structure. C) The binding site for DOX1 (ΔG^o = -9.1 kcal/mol). D) The binding site for DOX9 (ΔG^o = -7.0 kcal/mol).

Table S1: Comparison of PK Parameters between SPEDOX-6 and DOX*		
PK parameters	**SPEDOX-6**	**DOX**
Initial Phase $t_{1/2}$ (h)	0.426	0.069
Terminal Phase $t_{1/2}$ (h)	6.2	11
*AUC (h*ug/mL)*	82.54	1.74
C_{max} (ug/mL)	65.9	13
t_{max} (h)	0.083	0.083

*At 12 mg/kg DOX or DOX equivalent IV injection.

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Table S2: Encapsulated DOX by SPEDOX-6 in Blood Samples*

Time (h)	Total DOX (ug/mL)	Encapsulated DOX (ug/mL)	Encapsulated DOX (%)	Encapsulated DOX after Correction (%)‡
0.0833	65.90	34.50	52.3%	***74.7%***
2	2.91	1.68	57.7%	***82.5%***
8	0.70	0.42	60.0%	***85.7%***
24	0.15	0.08	54.7%	***78.1%***

‡Protein recovery from Zeba desalting spin columns ranged from 70 to 90% according to the manufacturer's protocol. A 70% protein recovery rate was used for correction of % encapsulated DOX.

Table S3: Tumor Types Targeted for SPEDOX Treatment (normal/tumor tissue FcRn ratio > 1.6)*		
Tumor Abbr.	**Tumor Type**	**FcRn ratio**
ACC	Adrenocortical carcinoma	2.63
BLCA	Bladder urothelial carcinoma	1.81
BRCA	Breast invasive carcinoma	1.66
CESC	Cervical squamous cell carcinoma and endocervic adenocarcinoma	3.48
CHOL	Cholangiocarcinoma	1.75
DLBC	Lymphoid neoplasm diffuse large B- lymphoma	1.55
KICH	Kidney chromophobe	1.92
LUAD	Lung adenocarcinoma	1.62
LUSC	Lung squamous cell carcinoma	3.14
OV	Ovarian serous cystadenocarcinoma	2.30
PRAD	Prostate adenocarcinoma	1.64
UCEC	Uterine corpus endometrial carcinoma	1.61

*Data were obtained from GEPAI (Gene Expression Profiling Interactive Analysis)

http://gepia.cancer-pku.cn/.