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Supporting Information

for

Design of Tumor-Homing and pH-Responsive Polypeptide-Doxorubicin Nanoparticles with Enhanced Anticancer Efficacy and Reduced Side Effects

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Content

Materials

All molecular biology reagents were purchased from New England Biolabs, unless otherwise specified. All chemical reagents were purchased from Sigma Aldrich and used as received, unless otherwise specified. C26 murine colon carcinoma cell line was purchased from cell bank of Chinese Academy of Medical Sciences. Female BALB/c nude mice were purchased from Vital River Laboratories (Beijing, China) and accommodated in animal research facility of Tsinghua University.

Construction, expression and purification of ELP-C8 and F3-ELP-C8

The gene encoding ELP-C8 was constructed into pET-25b(+) vector (Novagen) according to the literature.[1] The peptide sequence is SKGPG$(XGVPG)_{160}$WPC$(GGC)_7$ where the guest amino acid [X] composed of Val, Ala and Gly in the ratio of 1:8:7. To ligate F3 peptide (KDEPQRRSARLSAKPAPPKPE PKPKKAPAKK) into ELP-C8 in pET-25b(+), an 99-mer sense oligonucleotide (5'-TATGAAAGACGA ACCGCAGCGTCGTTCTGCTCG TCTG TCTGCTAAACCGGCTCCGCCGAAACCGGAACCGAAACCGAAAA AAGCTC CGGCTAAAAAACA-3') and 99-mer antisense oligonucleotide (5'-TATGTTTTTTAGCCG GAGCTTTTTTCGGTTTCGGTTCCGGTTTCGGCGGAGCCGGTTTAGCAGACAGACG A GCAGAACGACGCTGCGGTTCGTCTTTCA-3') were annealed and inserted into the *Nde* I digested vector using T4 DNA ligase.

The recombinant plasmid confirmed by DNA sequencing was transformed into *Escherichia coli.* BL21 (DE3) competents (Invitrogen) and incubated in Luria Bertani medium supplemented with ampicillin (100 μg/mL) at 37 ˚C. To express ELP, the culture was inoculated into a large scale of sterile Terrific Broth medium with shaking at 37 ˚C for 24 h.

Cells were harvested and resuspended in 50 mM Tris•HCl, 150 mM NaCl, pH = 7.4. After sonication and centrifugation, the extract was treated with polyethyleneimine (1% w/v) in order to precipitate nucleic acids, followed by centrifugation. The supernatant was heated to 60 °C for 10 min in a water bath, then 10 min in an ice bath and centrifuged at 16,000 x g at 4 °C for 15 min. The pellet was discarded and ELP in the supernatant was purified by inverse transition cycling (ITC), as described before[1] with minor modifications. The inverse phase transition was initiated by the addition of NaCl to a final concentration of 3 M and the aggregated ELP was separated from solution by centrifugation at 16,000 x g at 37 °C for 15 min. The pellet was resuspended in cold PBS buffer containing 10 mM Tris(2-carboxyethyl)phosphine (TCEP) (Strem Chemical Inc.) on ice for 15 min and centrifuged at 4 °C to remove any insoluble particles. This cycle was typically repeated 3 times. Purified ELP was stored in 100 mM Tris•HCl, 150 mM NaCl, pH = 7.0 at -80 °C for further use. The concentration of protein was estimated by UV-visible spectroscopy (Thermo Scientific) with the absorbance of 280 nm. The identity and purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation and characterization of ELP-C8-DOX and F3-ELP-C8-DOX

Drug conjugation

20 mL of 200 μM F3-ELP-C8 in 100 mM Tris•HCl, 150 mM NaCl, pH 7.0 was mixed with 2 mL of 100 mM Tris(2-carboxyethyl)phosphine (TCEP) (Strem Chemical Inc.) in Tris•HCl buffer at 25 °C. After 60 min, 10 mg of Maleimidopropionic acid hydrazide hydrochloride (BMPH) (J&K) in 1 mL Tris•HCl buffer was added into the reaction mixture, and the final reaction solution was allowed to sit without agitation overnight at room

temperature. The unreacted BMPH and TCEP were removed by initiating the phase transition with 3 M NaCl and centrifugation at 16,000 x g at 37 ˚C for 15 min. The aggregate was re-solubilized in 20 mL Tris•HCl buffer containing 100 mM aniline, 45 mg of doxorubicin (DOX) dissolved in 10 mL dimethyl sulfoxide (DMSO) was added dropwise, and the reaction was kept under stirring over 36 h at 25 ˚C in the dark. The product was further purified via ITC and dialysis to remove DMSO and unreacted DOX.

Efficiency of ligation

The concentration of protein was determined by bicinchoninic acid (BCA) assay according to the directions of BCA kit (Beyotime Biotech). The quantification of DOX was measured at the characteristic absorbance of 495 nm with the molar extinction coefficient of 1.00 x 10^4 M^{-1} cm^{-1} by Nanodrop 2000 (Thermo Scientific).

Dynamic light scattering (DLS)

DLS was carried out with a Malvern Zetasizer Nano-zs90. Samples were cleaned with a filter of 0.22 μm pore size before analysis. The instrument was operated at a laser wavelength of 633 nm and a scattering angle of 90° at 25 ˚C. All results were analyzed with Zetasizer software 6.32.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a SpectraMax M3 Microplate Reader (Molecular Devices) at 20 ˚C. The fluorescence of samples was measured at the excitation wavelength of 465 nm and the emission wavelength of 590 nm.

Transmission electron microscopy (TEM)

The morphology was characterized with transmission electron microscopy (Hitachi H-7650) operating at an acceleration voltage of 80 kV. A drop of sample (300 μM equivalent of

DOX) was applied on a TEM copper specimen grid covered with carbon film for 2 min and dried at room temperature.

Critical aggregation concentration (CAC)

The samples were diluted into different concentration (10, 8, 5, 4, 3, 2, 1, 0.8, 0.5 and 0.4 μM) and the fluorescence of each sample was measured.

Drug release

The samples were placed into the dialysis bags (10K MWCO, Spectrum Laboratories Inc.) and dialyzed in 10 mM PBS at pH 7.4 or pH 5.0 at 37 °C in the dark. The released free DOX in the dialysate at various time points (0, 0.25, 0.5, 1, 2, 4, 6, 8, 16 and 24 h) was measured by Nanodrop 2000 at the absorbance of 495 nm.

***In vitro* study**

Cellular uptake

C26 cells were cultured in RPMI-1640 medium (Gibco) containing 10% (vol/vol) fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Hyclone) at 37 ˚C in a humidified, 5% CO_2 atmosphere. C26 cells were seeded at 5 x 10^4 per well in 8-well Lab-Tek II chamber slides (Thermo Fisher Scientific) overnight for attachment. The attached cells were then incubated with 20 μM DOX equivalent of DOX, ELP-C8-DOX and F3-ELP-C8-DOX at 37 ˚C for different times (5, 15, 30, 45 and 60 min). After incubation, the cells were washed with PBS (Gibco) and fixed with 4% (wt/vol) cold paraformaldehyde. The cell membrane and nucleus were stained with 2 μg/mL of wheat germ agglutinin (WGA) Alexa Fluor 594 (Invitrogen) and 1 μg/mL bisBenzimide H 33342 trihydrochloride (Hochest33342) (Sigma) for 10 min, respectively. The cells were then washed with PBS three times and imaged with an LSM710 laser scanning confocal microscope (Carl Zeiss).The excitation and

emission wavelengths of Hochest, DOX and WGA were 346/460 nm, 485/590nm and 590/617 nm, respectively. Images were analyzed by Zen_2012 (blue edition) software.

The nucleolin expression in cell membrane was analyzed by immunofluorescence assay. The fixed cells were permeabilized with 0.4% Triton X-100 for 15 min and then blocked with 5% FBS for 60 min. After washed with PBS three times, the cells were incubated with C23 antibody-Alexa Fluor 488 (1:200, Santa Cruz Biotechnology) for 2 h at 37 ˚C. The cell membrane and nucleus were stained as described above.

***In vitro* cytotoxicity**

C26 cells were seeded in a 96-well plate at the density of 5,000 cells per well overnight. Media was removed and replaced with 100 μL DOX, ELP-C8-DOX or F3-ELP-C8-DOX diluted in fresh media at different serial concentrations (20, 10, 3.33, 1, 0.333, 0.1, 0.033, 0.01, 0.003 and 0.001 μM equivalent of DOX). Wells filled with media and media-treated cells only were background and control, defined as 0% and 100% cell viability, respectively. After incubating the plate for 48-72 h at 37 ˚C in a humidified, 5% CO_2 atmosphere, the viability of cells was measured by MTT assay according to Cell Proliferation Assay kit (Promega). The data fitting and IC_{50} calculation were made by GraphPad Prism 5.0 software and the data were presented as mean ± standard deviation.

***In vivo* study**

The Laboratory Animal Facility at the Tsinghua University is accredited by AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International), and all animal use protocols used in this study are approved by the Institutional Animal Care and Use Committee (IACUC).

Pharmacokinetics

The procedures of pharmacokinetics and biodistribution were employed as described previously[1] with slight modifications. Female BALB/c nude mice of 8 weeks old (n = 5 for each group) were used to determine the pharmacokinetics. 5 mg DOX Equiv/kg of DOX, ELP-C8-DOX and F3-ELP-C8-DOX were intravenously injected into mice. Blood samples were collected from the tail vein at selected time points (1, 5, 15, 30 min, 1, 2, 4, 8 and 24 h), standed for 30 min at 4 ˚C and centrifuged at 4000 x g for 15 min. 10 μL of obtained plasma was diluted into 190 μL of acidified isopropanol (0.075 M HCl, 10% water), incubated overnight at 4 ˚C and then centrifuged at 15,000 x g for 20 min. The pellet was discarded and DOX concentration in the supernatant was determined via fluorescence. To eliminate the background, the fluorescence of plasma from untreated mice was measured. A linear standard curve of DOX concentration ranging from 0.001 μM to 10 μM was created for the quantification of DOX concentration. Data was analyzed by DAS 3.0 software and fitted with a two-compartment model.

Biodistribution

Female BALB/c nude mice of 8 weeks old were inoculated subcutaneously in the left flank with 5 x 10^6 C26 cells. When the tumour volume reached 100-150 mm^3, mice were randomly assigned to three groups (n = 3 per group) and injected with 10 mg/kg DOX, ELP-DOX and F3-ELP-DOX. Tumour and major organs were obtained, weighed, homogenized and suspended in corresponding quantity of acidified isopropanol 6 h after injection. All homogenate samples were incubated at 4 ˚C overnight and centrifuged at 16,000 x g. The DOX concentration in supernatant was determined by Nanodrop 2000 at the absorbance of 495 nm. The tissue background from untreated mice was subtracted from the acquired data correspondingly. The results were presented as percentage of injected dose (%ID) per gram of

organ.

***In vivo* anti-tumour activity**

40 female BALB/c nude mice bearing C26 tumours of 100 mm^3 in size were randomly divided into 6 groups (n = 5-9 in each group). Each group received intravenous injection on day 0 and day 3 with a single dose of 10 mg/kg DOX, 10 mg/kg DOX equivalent ELP-DOX or F3-ELP-DOX, 1.5 mg/kg ELP-C8, 1.5 mg/kg ELP-C8, or saline. Tumour volumes and body weights of the mice were measured at 3-4 day intervals twice a week, and the tumour volume was calculated as volume = length x $width^2$ x 0.5. The mice were sacrificed if their tumour sizes grew up to 1000 mm^3 or weight loss was greater than 15% of body weight. Statistics were carried out by GraphPad Prism 5.0 software.

Histological examination

After tumours grew to a size of approximately 100 mm^3, the female BALB/c nude mice were treated with 10 mg/kg DOX, ELP-C8-DOX, F3-ELP-C8-DOX or saline on day 0 and day 3, and killed on day 6. Tumours and hearts were collected, fixed with 4% neutral paraformaldehyde and embedded in paraffin for histological examination. 5 μm thick tissue sections mounted onto glass slides were stained with hematoxylin-eosin (H&E) staining for morphology observation according to standard procedure. The images of all sections were captured with a Nikon Eclipse 90i microscope. Apoptotic events were determined by TdT-mediated dUTP nick end labeling (TUNEL) assay according to the instruction of TUNEL kit (Keygen Biotech) and imaged with an LSM710 laser scanning confocal microscope (Carl Zeiss).

References

[1] J. A. MacKay, M. N. Chen, J. R. McDaniel, W. G. Liu, A. J. Simnick, A. Chilkoti, *Nat. Mater.* **2009**, *8*, 993.

Fig. S1-S7

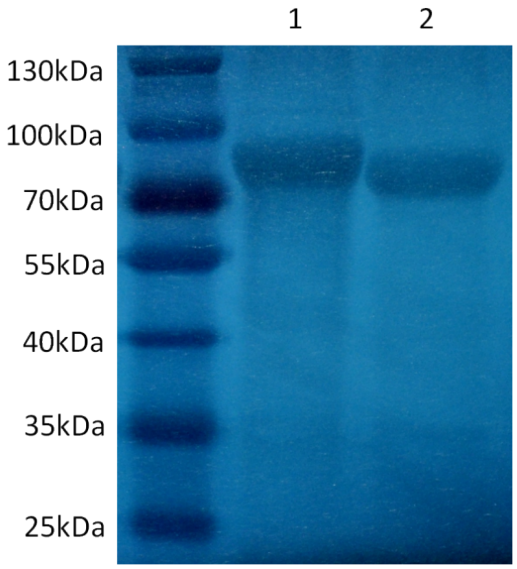

Fig. S1 SDS-PAGE analyses of F3-ELP-C8 (lane 1) and ELP-C8 (lane 2) after purification by ITC. The gel was stained with $CuCl_2$.

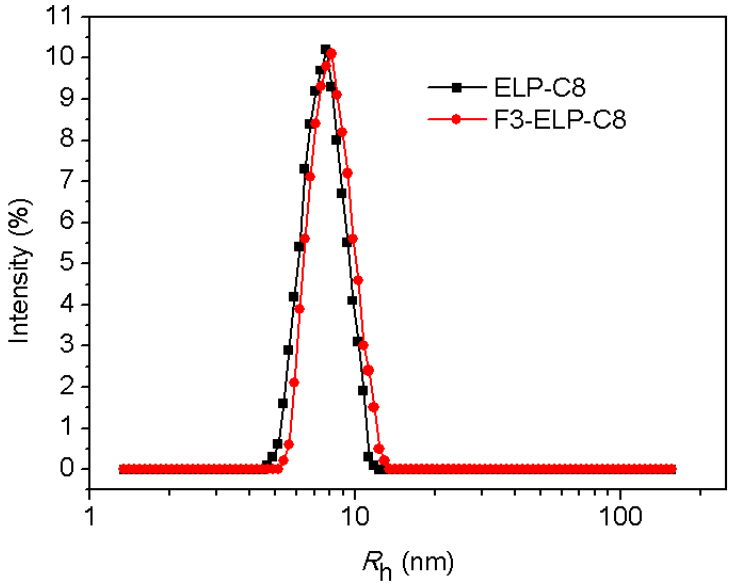

Fig. S2 DLS analysis of F3-ELP-C8 and ELP-C8, where R_h is hydrodynamic radius.

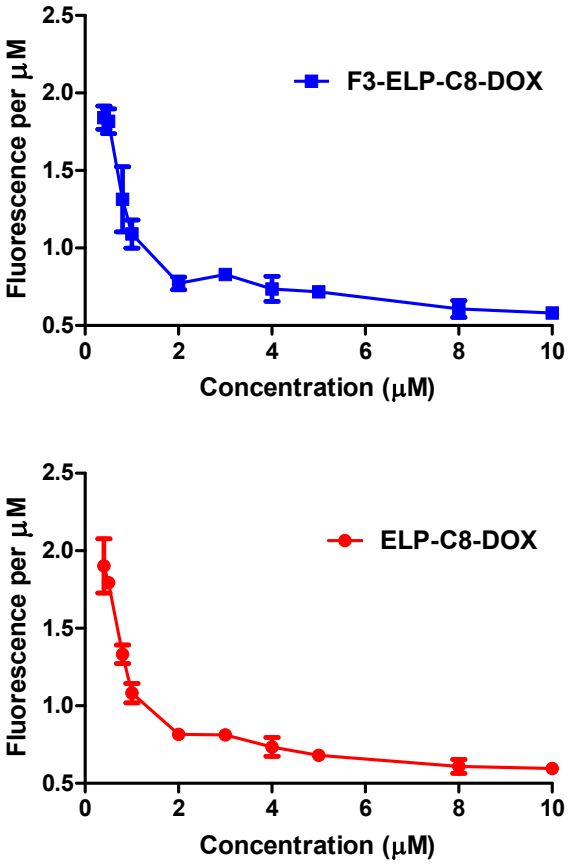

Fig. S3 The fluorescence as a function of concentration of F3-ELP-C8-DOX (left panel) or ELP-C8-DOX (right).

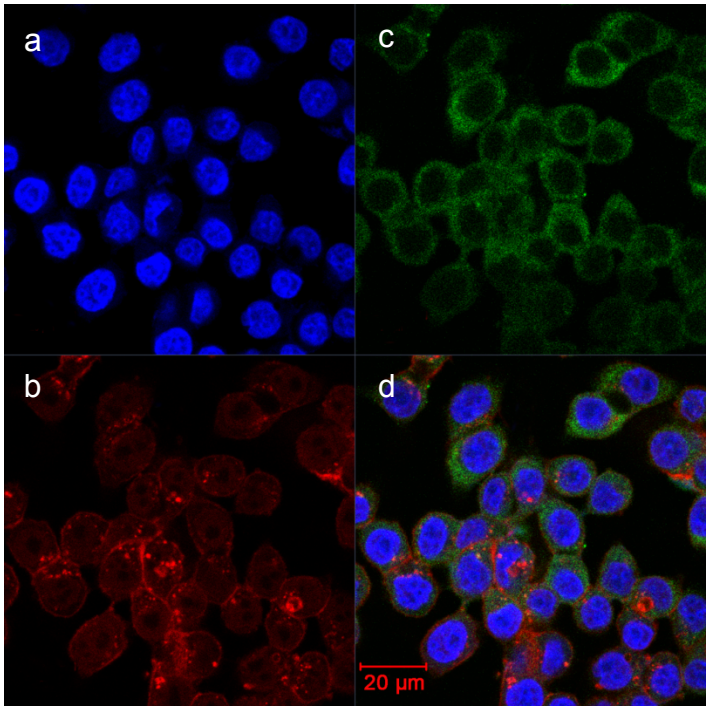

Fig. S4 The nucleolin expression on C26 cell membrane. Cell nucleus was stained with Hochest33324 in blue (a); cell membrane was stained with wheat germ agglutinin (WGA) in red (b); nucleolin was incubated with secondary C23 antibody-Alexa Fluor 488 in green (c); merged image (d).

Table S1 Pharmacokinetic parameters of DOX, ELP-C8-DOX, F3-ELP-C8-DOX.

Parameters	**DOX**	**ELP-C8-DOX**	**F3-ELP-C8-DOX**
distribution half-life t1/2α (h)	0.11 ± 0.04	0.17 ± 0.05	0.23 ± 0.05
terminal half-life t1/2β (h)	5.31 ± 2.67	10.95 ± 2.11	10.88 ± 1.64
central compartment volume of distribution V1 (L/kg)	436.97 ± 83.01	56.96 ± 1.64	58.92 ± 3.08
peripheral compartment volume of distribution V2 (L/kg)	2100.20 ± 889.94	105.97 ± 12.90	92.52 ± 8.29
central clearance CL1 (L/h/kg)	372.72 ± 57.31	10.64 ± 1.73	10.00 ± 1.47
peripheral clearance CL2 (L/h/kg)	2088.06 ± 638.07	143.83 ± 48.69	104.94 ± 19.21
area under curve AUC(0-∞) (uM/L*h)	13.82 ± 1.36	512.12 ± 79.84	540.00 ± 106.89
elimination rate constant K10 (1/h)	0.85 ± 0.12	0.19 ± 0.03	0.17 ± 0.02
central to peripheral rate constant K12 (1/h)	4.78 ± 2.30	2.53 ± 0.86	1.78 ± 0.35
peripheral to central rate constant K21 (1/h)	0.99 ± 0.44	1.36 ± 0.64	1.13 ± 0.29

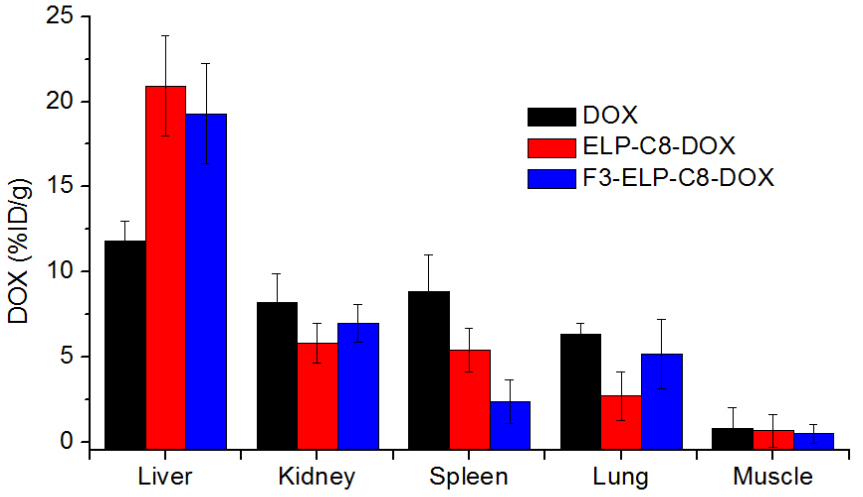

Fig. S5 Distribution of DOX in tissues 6 h post administration. Data are shown as mean ± standard deviation (n=3, $p < 0.01$).

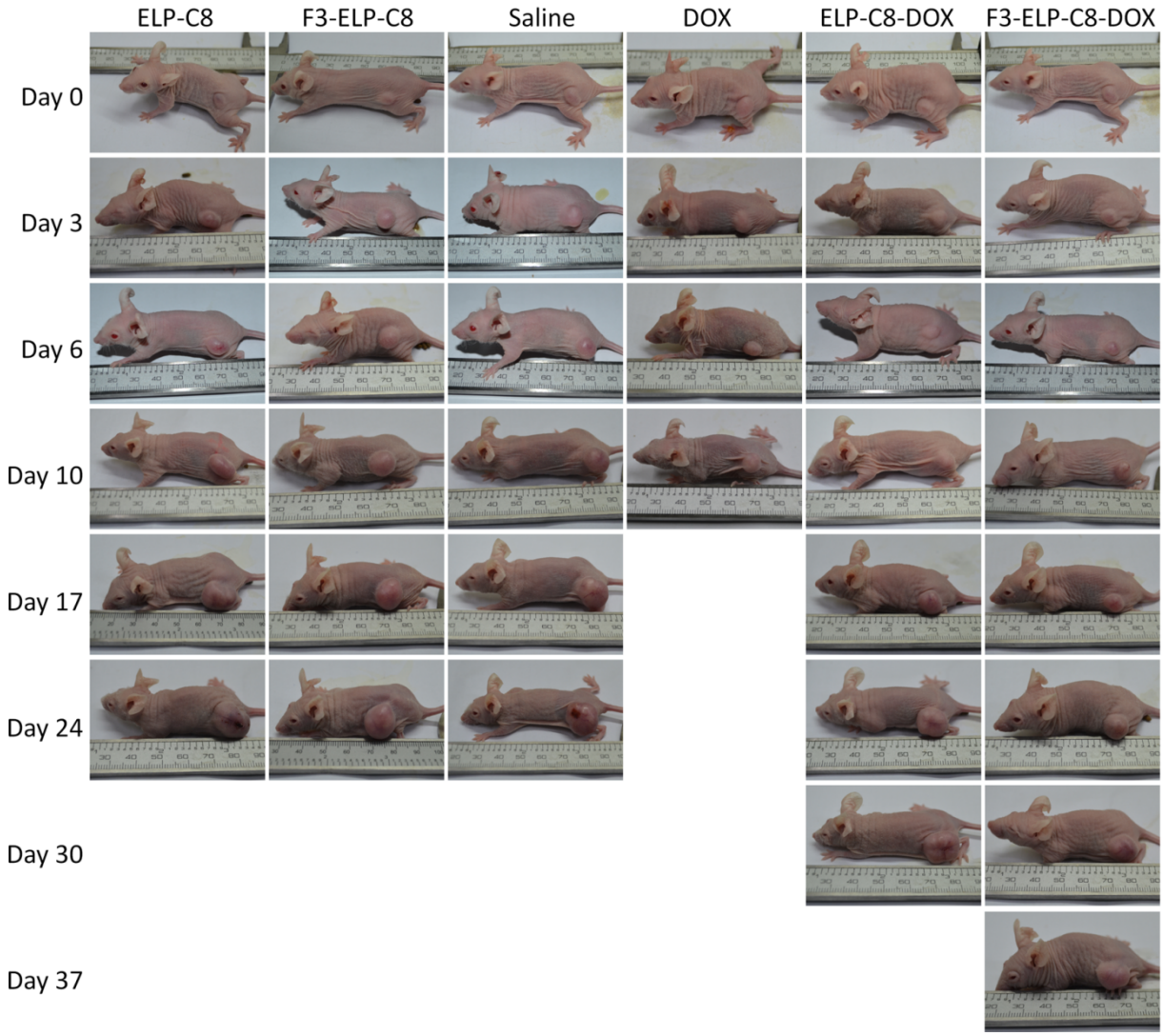

Fig. S6 Representative images for the tumor growth post administration.

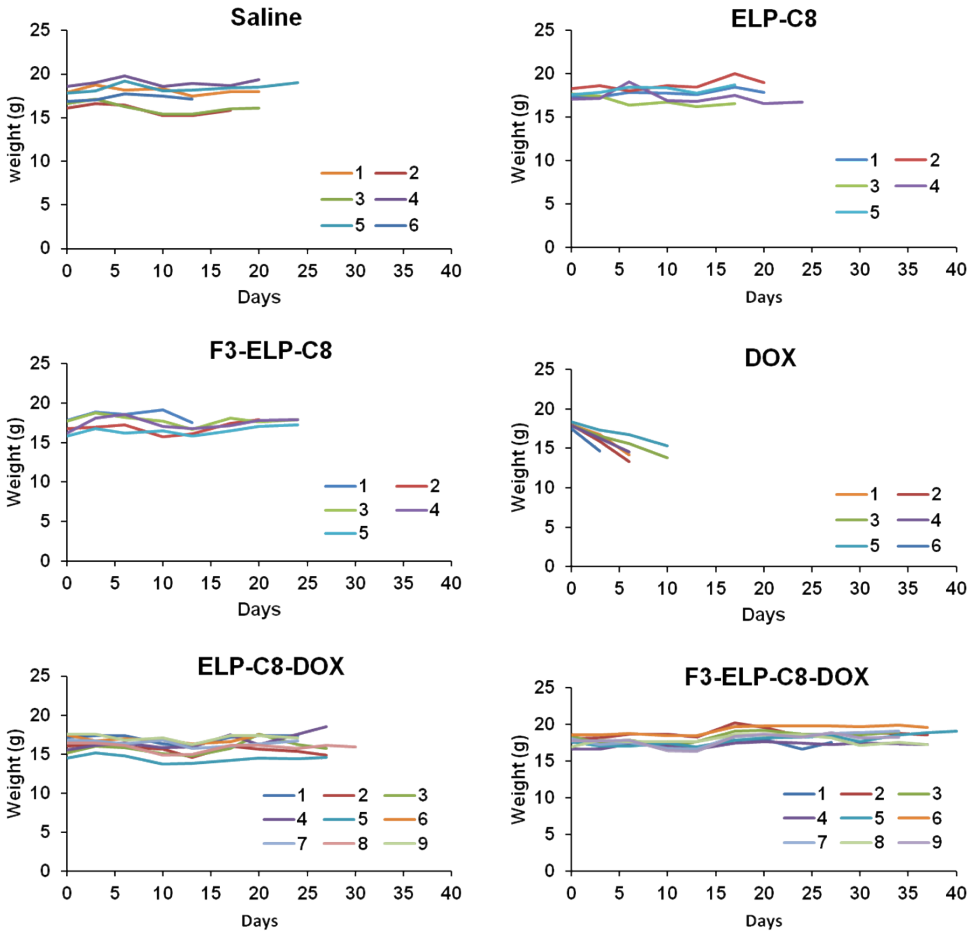

Fig. S7 The change of mouse body weight post administration.