Supplementary Materials

Multivalent Targeting Based Delivery of Therapeutic Peptide using AP1-ELP Carrier for Effective Cancer Therapy

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Design of AP1-ELP-KLAK

The pET 25 b+ (Novagen, Canada, USA) expression plasmid was modified by ligating annealed oligonucleotides (forward : T ATG AGC GGG CCG GGC TGG CCG GGC GGC AGC AAA CTG GCG AAA CTG GCG AAA AAA CTG GCG AAA CTG GCG AAA TGC TAA A, reverse : AGCTT TTA GCA TTT CGC CAG TTT CGC CAG TTT TTT CGC CAG TTT CGC CAG TTT GCT GCC GCC CGG CCA GCC CGG CCC GCT CA) encoding $(KLAKLAK)_2$ sequences flanked by *Nde* I and *Hin* DIII restriction sites. After confirmation by DNA sequencing, the modified pET 25b+ vector was linearized with *Sfl* I and dephosphorylated with CIP enzymes to inhibit self-ligation. $[AP1\text{-}V_{12}]_6$ genes, synthesized previously by the recursive directional ligation method, was ligated into the linearized vector after *Pfl* MI and *Bgl* I digestion and named AP1-ELP-KLAK. For the control ELP, synthetic oligonucleotides encoding the monomer gene ELP $(V_3G_3A_1)_n$ [where "*n*" refers to the number of monomer gene repeats with respective guest residues Val, Gly, and Ala] were first ligated into pRSET B+ cloning vector with *Bam* HI and *Hin* DIII restriction sites. After two rounds of RDL, the resulting vector containing $(V_3G_3A_1)_3$ was digested with *Pfl* MI and *Bgl* I and ligated into *Pfl* MI linearized vector containing the monomer gene of ELP (V_7). The resultant gene $(V_3G_3A)_3\text{-}V_7$ was used as a monomer gene to perform two additional rounds of RDL. $[(V_3G_3A_1)_3\text{-}\ V_7]_3$ gene, obtained after two rounds of RDL was finally ligated into modified pET 25b+ vector consisting of KLAK sequence and designated as ELP-KLAK. Plasmids containing respective gene ligations were confirmed by restriction digestion with *Nde* I and *Hin* DIII, followed by gene sequencing (Macrogen Inc., Seoul, Korea). All restriction endonucleases were obtained from New England Biolab, Ipswich, MA.

In vitro Plasma stability

The stability of polypeptides in forming micelle like structure was determined in mouse plasma. 50 μM of ELP-KLAK and AP1-ELP-KLAK were incubated with 100% mouse plasma for different time interval (0, 6, 12 and 24 h) at 37°C. At the end of the incubation period, the protein sample were diluted with PBS and subjected to measure optical density by UV-Visible spectrophotometer together with particle size by DLS. Further proteolysis of polypeptides in plasma was examine through copper chloride staining following SDS-PAGE. Degradation was determined compared with controls samples such as polypeptides in plasma (immediately mixed and diluted with PBS) or protein diluted in PBS.

Competition and inhibition of KLAK containing polypeptide mediated apoptosis

MDA MB231 cells were co-incubuted with different concentration of anti-IL4R (5, 10 μg) or endocytosis inhibitors such as Dansylcadaverine (50 μM) and Genistein (100 μM) along with KLAK containing polypeptides (10 μM) for 4 h. Both floating and adherent cells were harvested, washed with PBS. Further, 2 μg mL^{-1} of propidium iodide (PI) and 5 μL of FITC annexin V reagent were added to stain necrotic and apoptotic cells. Annexin V and PI-positive cells were measured using flow cytometry. Data are represented as mean ± s.d. (n = 5). *P<0.01 and NS : not significant, (Student's t-test).

Fluorophore conjugation

ELP-KLAK and AP1-ELP-KLAK proteins were labeled with Alexa Fluor 488-C5 maleimide (Invitrogen) and Flamma FPR-675 (BioActs) for in vitro and in vivo studies. Briefly the protein samples of 100 μM were reacted with 10 molar excess of Alexa Fluor 488-C5 maleimide or

Flamma FPR-675 at 4°C for overnight. Unconjugated dyes were removed through Sepadex G-25 Column (GE Healthcare, UK) followed by concentrated using Amicon Ultra centrifugal filters (Ultracel 10K). Degree of labeling was estimated as described in company's protocol.

ELP-KLAK

MSGPGVGVPGGGVPGGGVPGVGVPGAGVPGVGVPGGGVPGVGVPGGGVPGGGVPGV
GVPGAGVPGVGVPGGGVPGVGVPGGGVPGGGVPGVGVPGAGVPGVGVPGGGVPGVG
VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGGGVPGGGVPGVGVPGAGV
PGVGVPGGGVPGVGVPGGGVPGGGVPGVGVPGAGVPGVGVPGGGVPGVGVPGGGVP
GGGVPGVGVPGAGVPGVGVPGGGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPG
VGVPGVGVPGGGVPGGGVPGVGVPGAGVPGVGVPGGGVPGVGVPGGGVPGGGVPGV
GVPGAGVPGVGVPGGGVPGVGVPGGGVPGGGVPGVGVPGAGVPGVGVPGGGVPGVG
VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGWPGGS**KLAKLAKKLAKLAK**C

AP1-ELP-KLAK

MSGPGVG**RKRLDRN**GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV
GVPGVGVPGVGVPGVGVPGVG**RKRLDRN**GVGVPGVGVPGVGVPGVGVPGVGVPGVG
VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVG**RKRLDRN**GVGVPGVGVPGVGV
PGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVG**RKRLDRN**
GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPG
VGVPGVG**RKRLDRN**GVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGV
GVPGVGVPGVGVPGVGVPGVG**RKRLDRN**GVGVPGVGVPGVGVPGVGVPGVGVPGVG
VPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGVGVPGWPGGS**KLAKLAKKLAKLAK**C

Fig. S1. Amino acid sequences of ELP-KLAK and AP1-ELP-KLAK. Red: IL-4R targeting peptide (AP1), Blue: Proapoptotic peptide, KLAK.

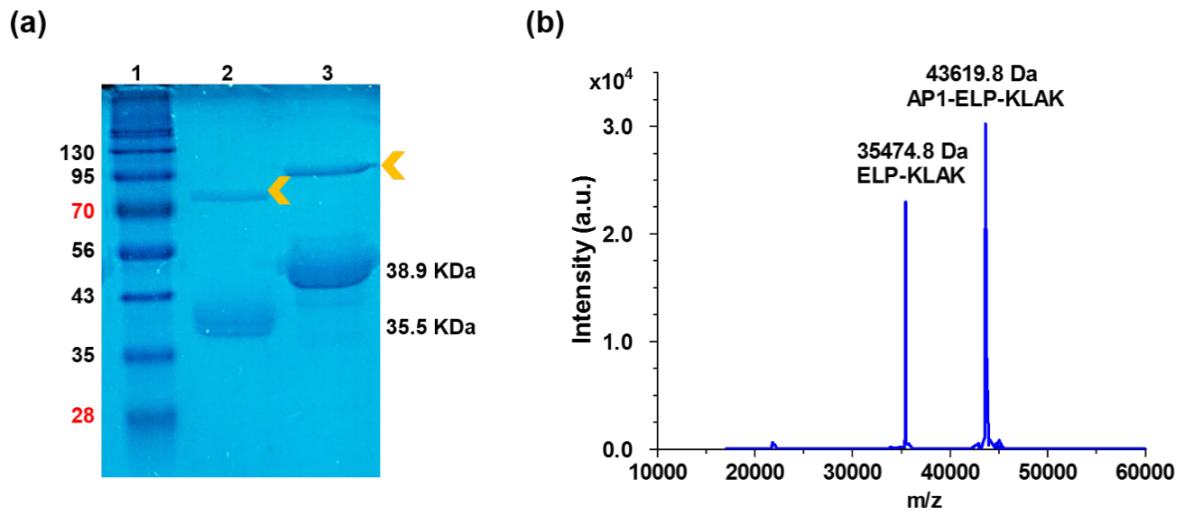

Fig. S2. (**a**) SDS–PAGE analysis of ELP-KLAK and AP1-ELP-KLAK stained with copper chloride. From left, lane 1: Prestained protein marker; lane 2: ELP-KLAK (~35.5 KDa); lane 3: AP1-ELP-KLAK (~38.9 KDa). Yellow: Upper band indicates dimer form of protein due to presence of cysteine residue. (**b**) MALDI-TOF MS spectra of ELP-KLAK and AP1-ELP-KLAK.

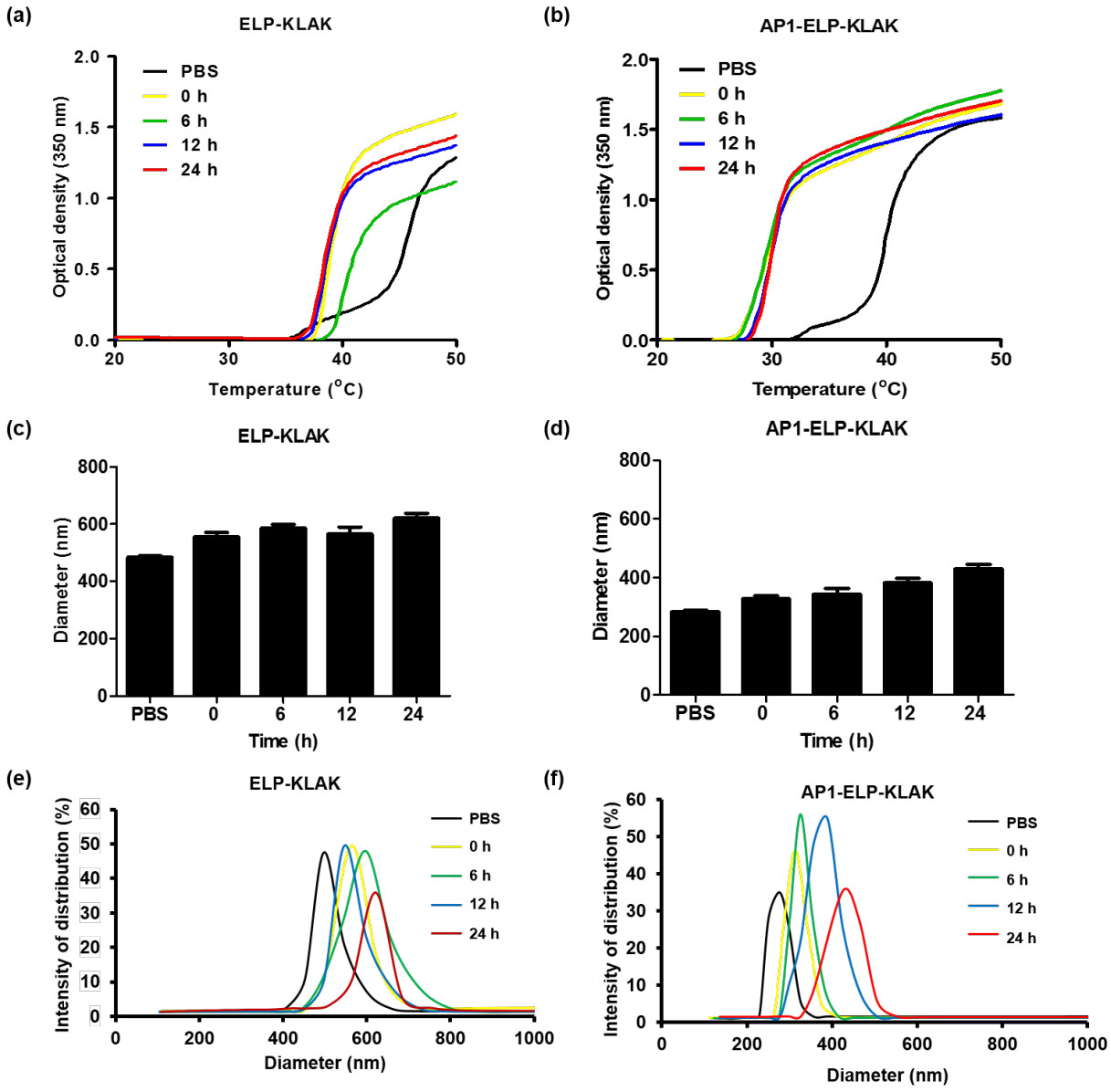

Fig. S3. Stability of micelle forming ELP-KLAK and AP1-ELP-KLAK in plasma. (**a**) ELP-KLAK and (**b**) AP1-ELP-KLAK (50 µM) were incubated with 100% mouse plasma for different time interval (0, 6, 12 and 24 h) at 37°C. At the end of incubation the protein sample were diluted with PBS, optical density of the particles were measured. **(c, d)** Similarly the size of the nanoparticle formed by both polypeptides were determined using DLS. **(e, f)** Hydrodynamic diameter (major peak) by number distribution of ELP-KLAK and AP1-ELP-KLAK at end of incubation.

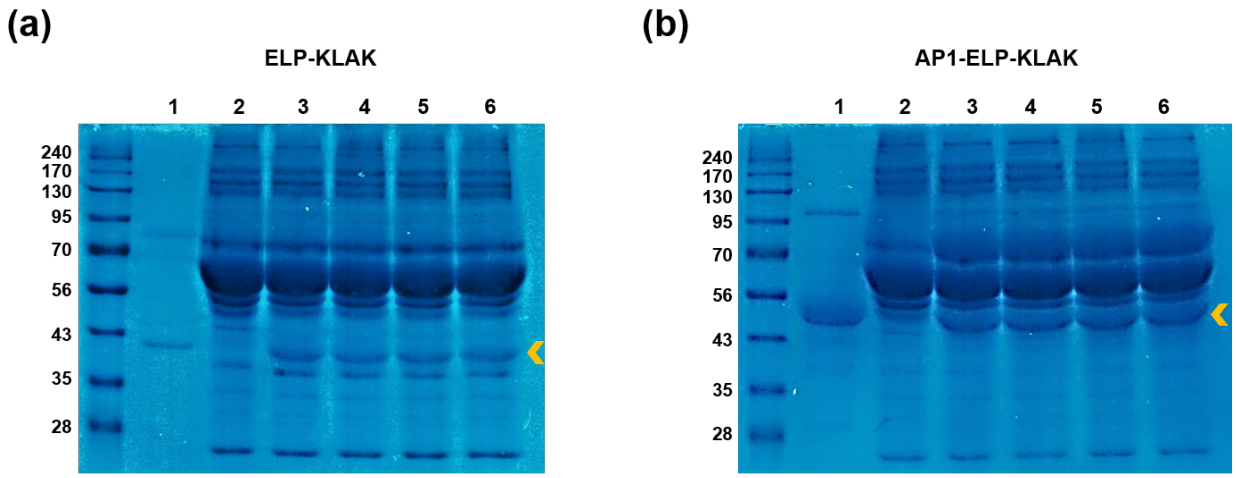

Fig. S4. Plasma stability of (a) ELP-KLAK and (b) AP1-ELP-KLAK. SDS–PAGE analysis stained with copper chloride showing the stability of ELP-KLAK and AP1-ELP-KLAK in 100% mouse plasma. From left, lane 1: protein in PBS, lane 2: plasma only; lane 3: protein in plasma (0 h); lane 4: protein in plasma (6 h); lane 5: protein in plasma (12 h); lane 6: protein in plasma (24 h). Yellow arrow heads indicate the presence of ELP-KLAK and AP1-ELP-KLAK in respective gels.

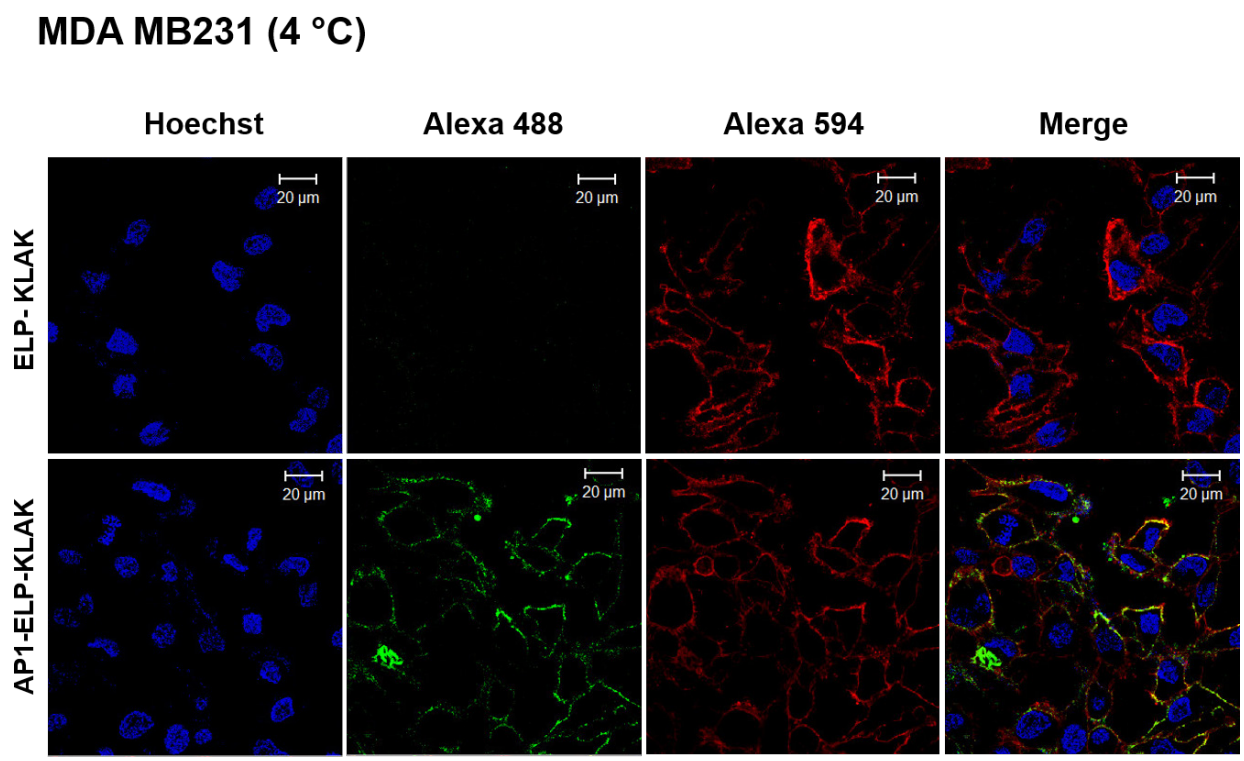

Fig. S5. Cellular accumulation and internalization of AP1-ELP-KLAK. MDA MB231 was treated with ELP-KLAK and AP1-ELP-KLAK (10 µM) for 1 h at 4 °C. After cells were fixed with 4% paraformaldehyde, cell membranes and nuclei were stained with WGA Alexa 594 and Hoechst. Representative confocal images of three experiments (scale bar 20 µm).

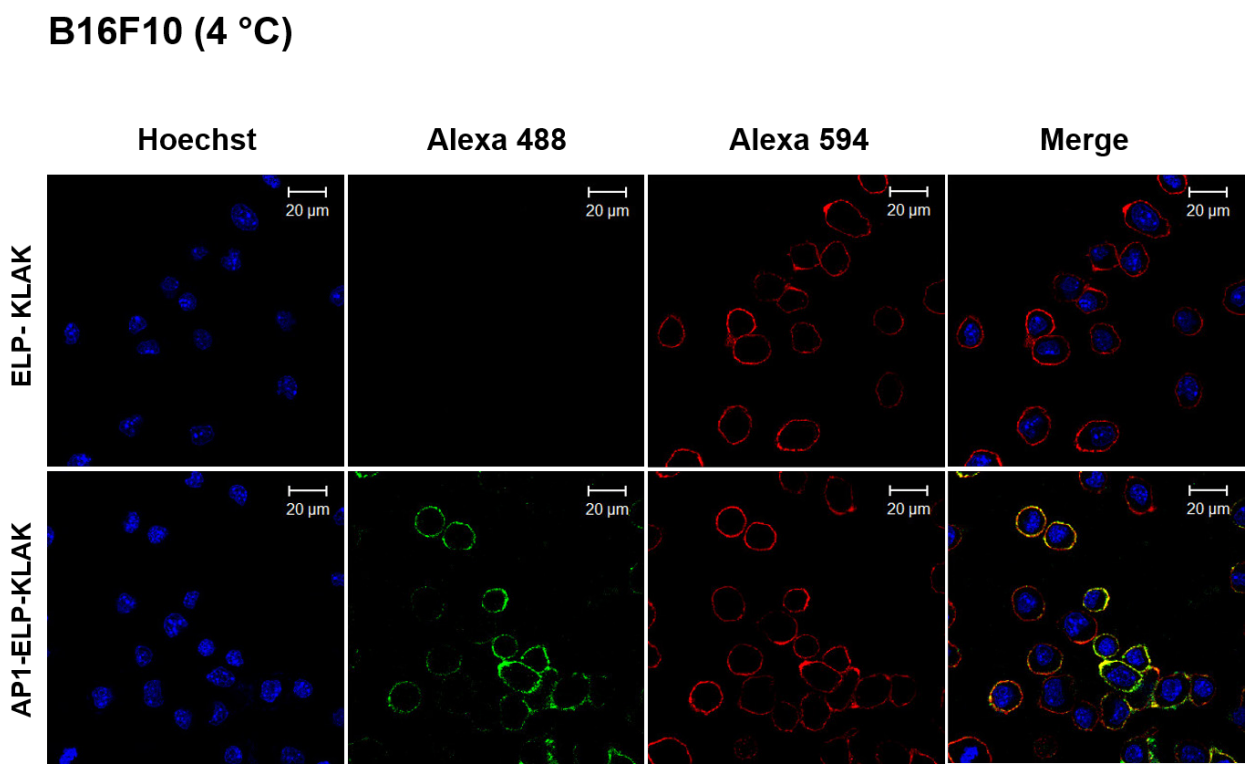

Fig. S6. Cellular accumulation and internalization of AP1-ELP-KLAK. B16F10 cells were treated with ELP-KLAK and AP1-ELP-KLAK (10 µM) for 1 h at 4 °C. After cells were fixed with 4% paraformaldehyde, cell membranes and nuclei were stained with WGA Alexa 594 and Hoechst. Representative confocal images of three experiments (scale bar 20 µm).

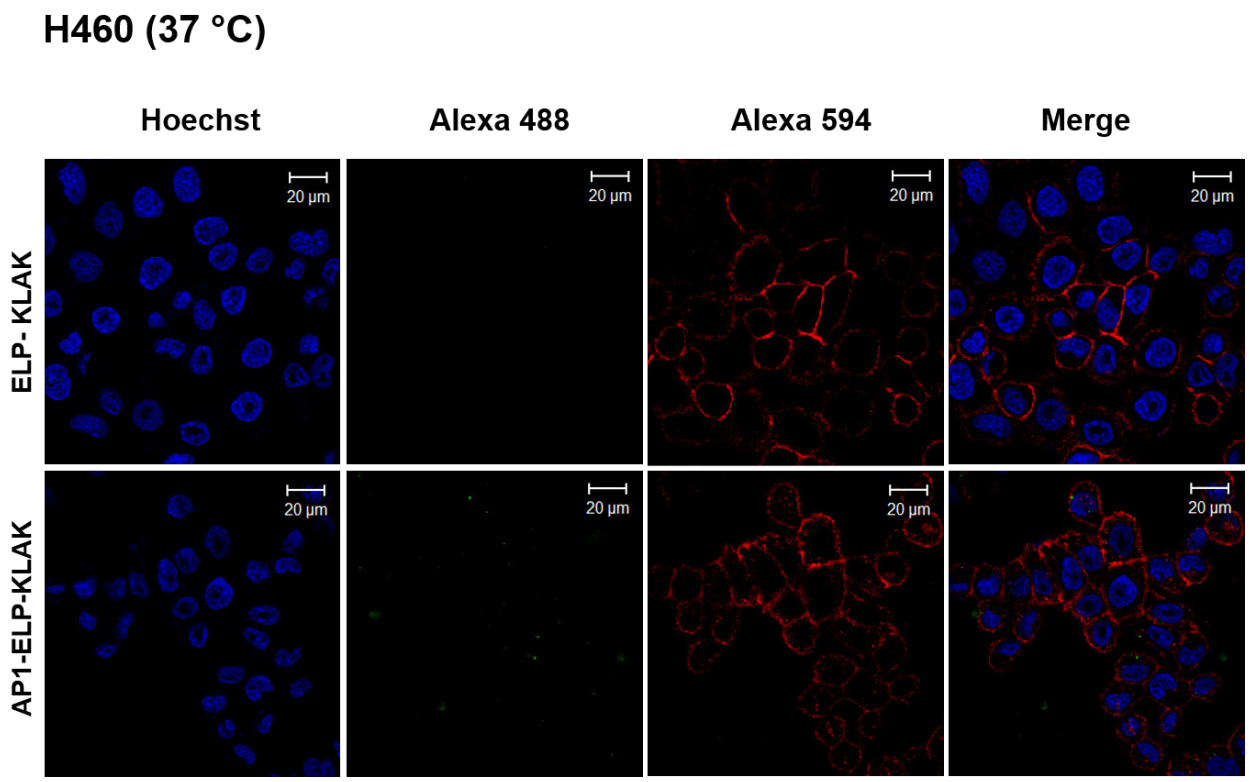

Fig. S7. Confocal images of H460 cells treated with respective polypeptides at 37 °C. Cell membranes and nuclei were stained with WGA Alexa 594 and Hoechst. Representative confocal images of three experiments (scale bar 20 µm).

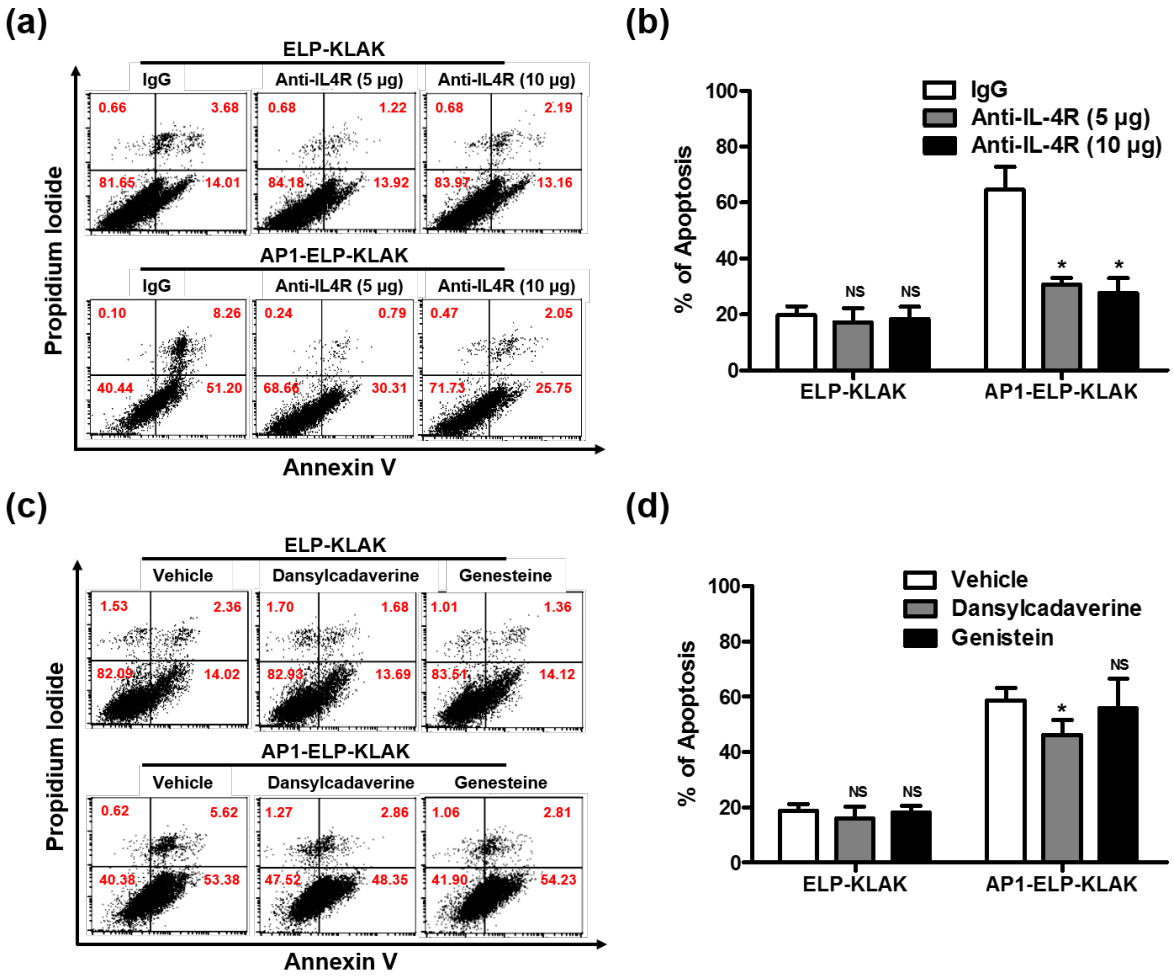

Fig. S8. Competition and inhibition of KLAK containing polypeptide mediated apoptosis. MDA MB231 cells were co-incubuted with different concentration of (a,b) anti-IL-4R and (c,d) endocytosis inhibitors (Dansylcadaverine and Genistein) with KLAK containing polypeptides (10 μM) for 4 h, and level of apoptosis was measured by flow cytometry. Data are represented as mean ± s.d. (n = 5). *P<0.01 and NS: not significant, (Student's t-test).

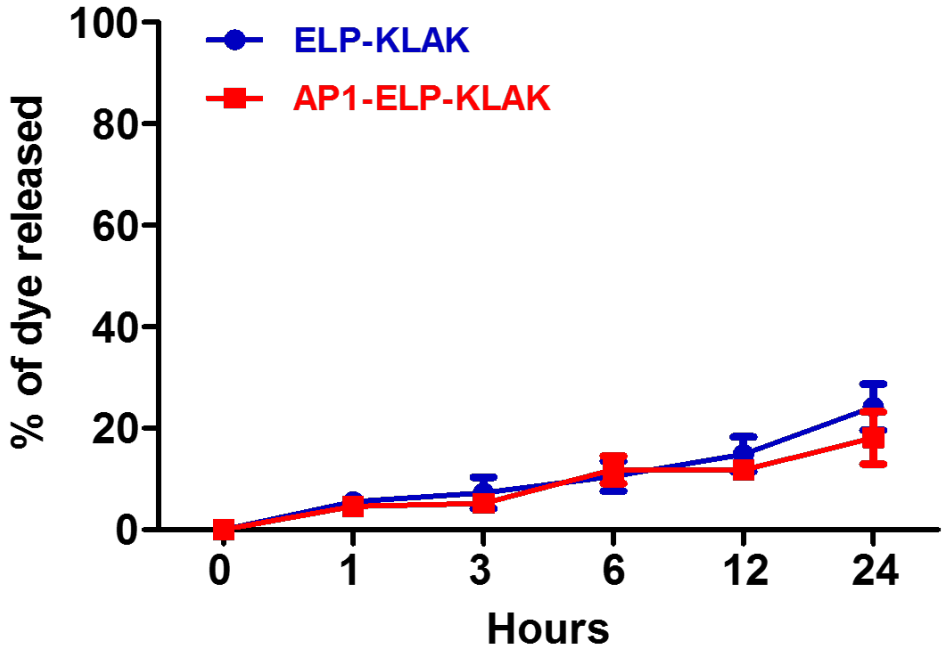

Fig. S9. Plasma stability and biodistribution. Stabilities of ELP-KLAK and AP1-ELP-KLAK conjugated with FPR-675 Flamma were determined after incubation with plasma at 37 °C. Dye released in plasma was measured by a spectrophotometer after two rounds of ITC. Data are represented as mean s.d. (n = 6).

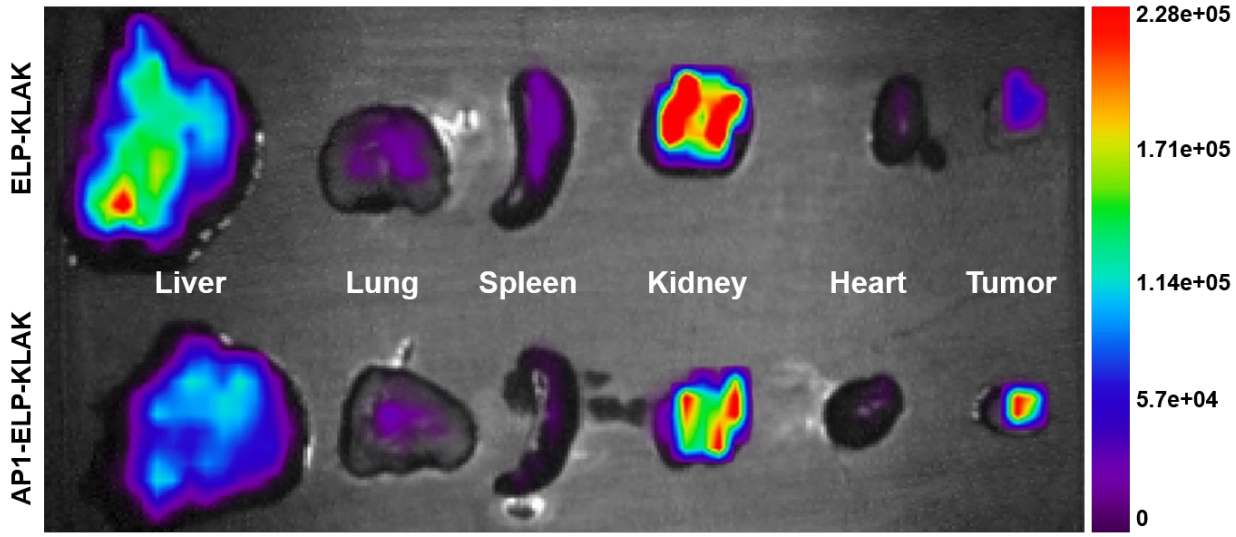

Fig. S10. MDA MB231 tumor bearing mice were IP-injected with ELP-KLAK and AP1-ELP-KLAK labeled polypeptides (n = 10). Ex vivo fluorescence images of organs and tumor tissues were obtained at 6 h post-injection. Scale bars indicate normalized fluorescence intensity.

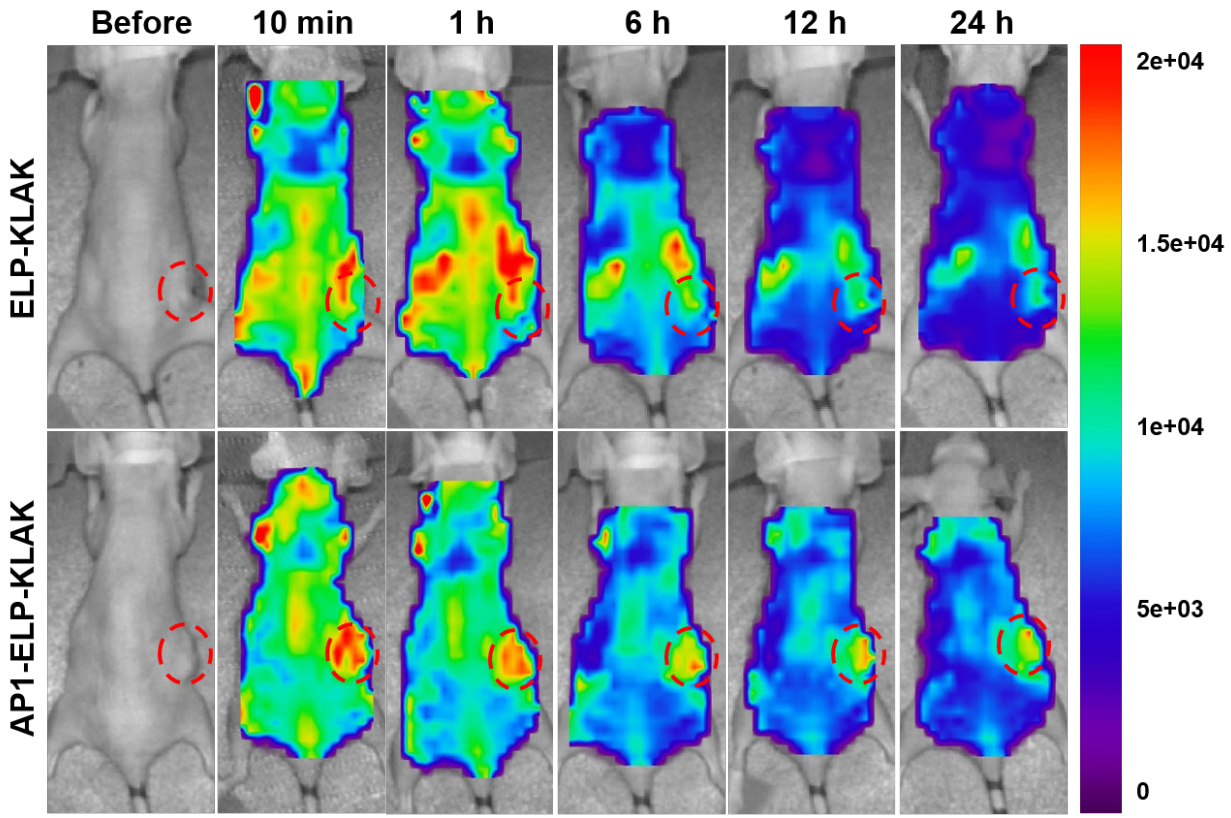

Fig. S11. Biodistribution of AP1-ELP-KLAK. (a) FPR 675-labeled ELP-KLAK and AP1-ELP-KLAK (150 mg kg^{-1}) were injected intravenously (IV) into MDA MB231 tumor xenograft nude fluorescence images were collected at different time points of labeled polypeptides. Scale bars indicate normalized fluorescence intensity. Representative optical images of ten experiments.

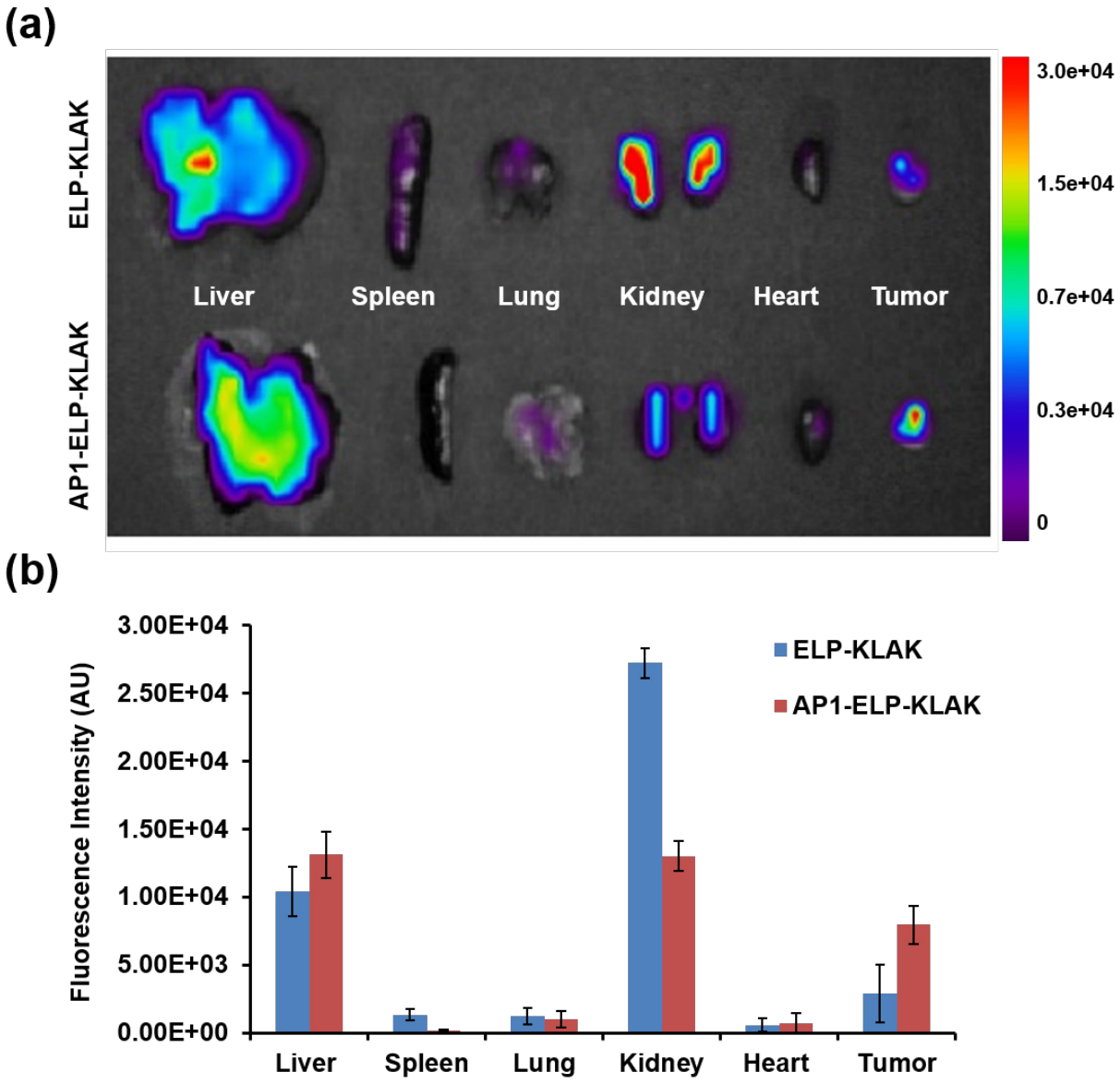

Fig. S12. Biodistribution of AP1-ELP-KLAK. FPR 675-labeled ELP-KLAK and AP1-ELP-KLAK (150 mg kg^{-1}) were injected intravenously (IV) into MDA MB231 tumor bearing mice. (a) *Ex vivo* fluorescence images of organs and tumor tissues were obtained at 6 h post-injection. Scale bars indicate normalized fluorescence intensity, (b) *ex vivo* images quantification data. (n=10).

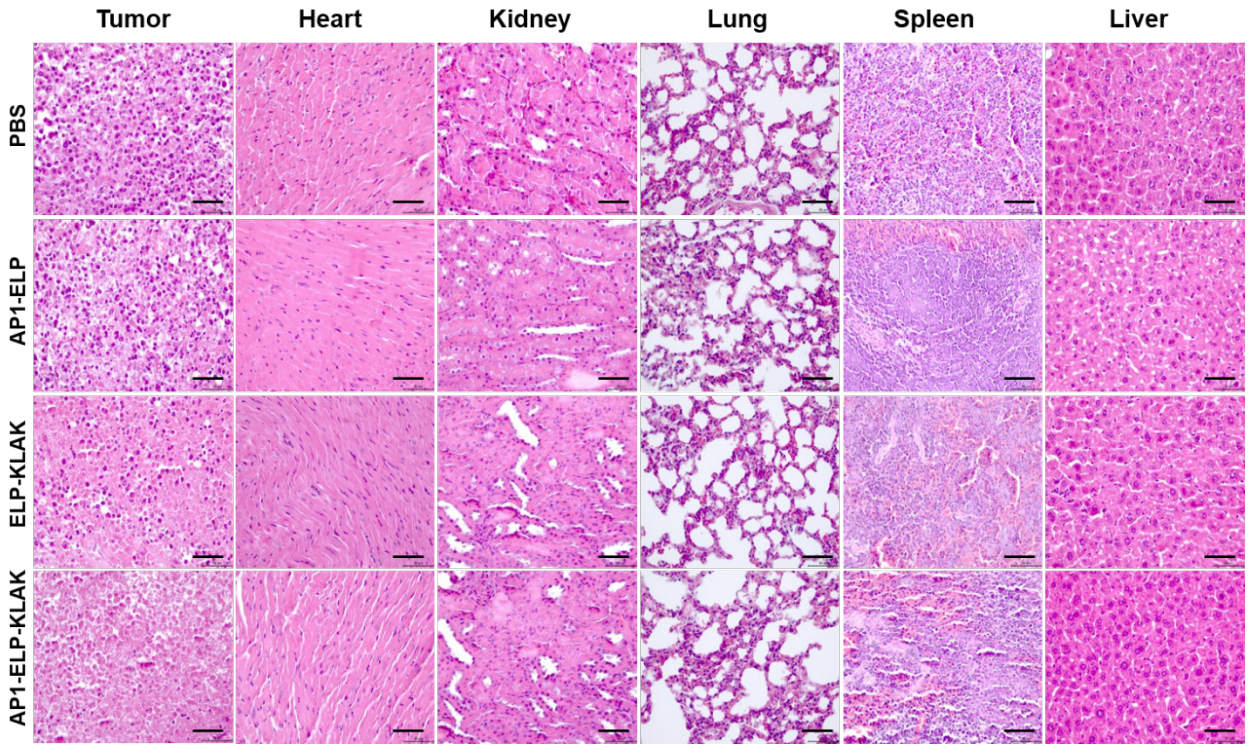

Fig. S13. H & E staining of major organs (heart, spleen, lung, kidney, and liver) along with tumor tissues extracted from MDA MB231 xenograft mice after therapy. Images were taken using light microscopy at 40X magnification. Representative images of six experiments (Scale bar, 50 μm).

Table S1. Secondary structural components ratio obtained from measured CD spectrum (residue molar ellipticity).

Parameters	KLAK peptide	AP1-ELP	ELP-KLAK	AP1-ELP-KLAK
Helix	0	15.5	26.1	30.2
Beta	2.9	24.4	0	0
Turn	26.3	0	0	0
Random	70.9	60.1	73.9	69.8

Table S2. Plasma pharmacokinetic parameters of ELP-KLAK and AP1-ELP-KLAK. Plasma clearance of labeled polypeptides were analyzed using Non-compartmental pharmacokinetic model.

Parameters	ELP-KLAK	AP1-ELP-KLAK
$T_{1/2}$ (h)	17.96 ± 2.25	14.40 ± 1.2
C_{max} (μg/ml)	3550±220	3890±145
AUC (h*μg/ml)	16290.64±2102	15690.86±1433
V_d (L/kg)	0.14± 0.01	0.13± 0.02
Cl (L/hr/kg)	0.0042± 0.0002	0.0046 ± 0.0007

($T_{1/2}$: terminal half-life; C_{max}: maximum plasma protein concentration; AUC: area under plasma concentration time curve; V_d: volume of distribution; Cl: plasma clearance. Values represented as mean ± s.d., n = 8).