Supporting Information for

ORIGINAL ARTICLE

Actively priming autophagic cell death with novel transferrin receptor-targeted nanomedicine for synergistic chemotherapy against breast cancer

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1. Materials and methods

1.1. Materials

The polymers of *N*-hydroxysuccinimidyl-PEG_{2000}-$DSPE_{800}$ (MW 2986) and PEG_{2000}-$DSPE_{800}$ (MW 2922) were obtained from NOF Corporation (Tokyo, Japan). Histidine-alanine-isoleucine-tyrosine-proline-argnine-histidine (7pep, MW 892.5) was synthesized by China Peptides Co., Ltd (Shanghai, China). Paclitaxel (PTX) was from Haikou Pharmaceutical Co., Ltd. (Hainan, China). Rapamycin (RAP) was purchased from Ke Rui Co., Ltd (Fujian, China). Hoechst 33258 (λ_{ex}= 352 nm, λ_{em}= 460 nm) was from Molecular Probes Inc. (Eugene, USA). Near-infrared lipophilic carbocyanine DiR (λ_{ex}= 748 nm, λ_{em}= 780 nm) and DiD (λ_{ex}= 630 nm, λ_{em}= 700 nm) were obtained from Biotium, Inc. (Hayward, USA). Sulforhodamine B (SRB), 3-methyladenine (3-MA), Dansylcadaverine (MDC), anti-LC3B monoclonal antibody,

Mitotracker (λ_{ex}= 630 nm, λ_{em}= 570 nm), Tris base and coumarin-6 (C6, λ_{ex}= 467 nm, λ_{em}= 502 nm) were from Sigma-Aldrich (St. Louis, MO, USA). PE mouse antihuman CD71 (TfR) antibody and PE mouse IgG2a were products of BD Pharmingen (San Diego, CA, USA). Mitotracker deep Red (λ_{ex}= 630 nm, λ_{em}= 570 nm), BCA protein quantitation kit, Annexin V-FITC/PI apoptosis detection kit, TUNEL in situ cell death detection kit, LC3B and cytochrome C ELISA kits, Caspase-3, 8, 9 detection kits, and ATP bioluminescence assay kit were purchased from KeyGEN Biotech Co., Ltd (Nanjing, China). LC3B antibody and goat anti-rabbit IgG(H+L) were purchased from Beyotime Biotech Co., Ltd (Shanghai, China). Cyto-ID® autophagy detection kit was from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) (Cyto-ID dye, λ_{ex}= 488 nm, λ_{em}= 560 nm). Cell culture medium RPMI-1640 (pH 7.4), penicillin-streptomycin and 0.25% trypsin were purchased from M&C Gene Technology (Beijing, China). Fetal bovine serum was from GIBCO, Invitrogen Corp. (Carlsbad, CA, USA). All other solvents and reagents were of analytical grade.

MCF-7 human breast cancer cells were purchased from the Institute of Basic Medical Science (Beijing, China). MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL, penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere.

Female nu/nu nude mice of 18–20 g were purchased from Vital River Laboratory Animal Center (Beijing, China). Care and handlings of all mice adhered to the approval of Institutional Animal Care and Use Committee of Peking University.

1.2. Synthesis of functional copolymer 7pep-PEG-DSPE

7pep-PEG-DSPE was synthesized according to previous reports[1]. Briefly, NHS-PEG-DSPE and 7pep were reacted in DMF with pH 8.0 at 2:1 molar ratio. The reaction was maintained for 120 h under moderate stirring and monitored by Thin-Layer Chromatography (TLC). Then, the reacted mixture was dialyzed (MW cut off 3500 Da) to remove the unconjugated 7pep, the dialysate was freeze-dried. The conjugation efficiency was monitored by the high-performance liquid chromatography (HPLC, Shimadzu, Japan). The final product was characterized by

matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Waters, Milford, MA, USA) and ultraviolet-visible spectrophotometer (Beijing puxi, TU-1900, China).

1.3. Preparation and characterization of the functional nanocarriers

Functional nanocarriers were prepared by film hydration method. In brief, for 7pep modified nanomedicines, PEG-DSPE, 7pep-PEG-DSPE (21.0:10.3, *w/w*; 17:3, mol/mol), and drugs (PTX, RAP, C6, or DiR) were dissolved in dichloromethane. Then, the solvent was evaporated by vacuum rotary evaporation at 40 ºC to form a thin film. The obtained dry film was hydrated in PBS and vortexed for 20 seconds before water-bathing at 60 ºC for 1 h to form micelles. The unencapsulated drugs were removed by centrifugation at 10,000 rpm for 5 min and filtered through a membrane with a pore size of 0.22 μm. The weight ratio of polymers and drugs was 30:1 for PTX, 30:1 for RAP, 10000:1 for C6, and 3000:1 for DiR, respectively.

The particle size and zeta potential of freshly prepared and diluted micelles were determined in triplicate by a dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS (Malvern, UK) at 25 °C. The data from diluted nanomedicines were used to assess the dilution stability of micelles. The morphology of 7pep-M-PTX and 7pep-M-RAP was observed by transmission electron microscope (TEM, JEOL, JEM-1400, Japan) after 150 times dilution and negative staining with 1% phosphotungstic acid solution.

The encapsulation efficiency (EE) was calculated by the following formula: EE (%) = Drug loaded/Total drug × 100. The concentration of C6 or DiR was determined by a fluorescence spectrometer (Cary Eclipse, Varian Corporation, USA). The EE% of PTX and RAP in micelles was quantified by a HPLC system.

The PTX powder, lyophilized blank micelles, physical mixture of PTX with lyophilized blank micelles, lyophilized PTX-loaded micelles, RAP powder, physical mixture of RAP with lyophilized blank micelles, and lyophilized RAP-loaded micelles were characterized by an X-ray diffractometer (Dmax 2400, Rigaku Corporation, Japan) with Cu-Kα radiation (λ = 1.5406 Å) in the 2θ range from 4 ° to

50 ° in the normal routine with a 4 °/min scanning velocity and 0.02 º step at 40 kV and 100 mA. The slit widths were set at 1/2 ° for DS, 1/2 ° for SS and 0.3 mm for RS.

The critical micelle concentrations (CMC) of PEG-DSPE and 7pep-PEG-DSPE were determined using previously method[2]. Briefly, a predetermined volume of polymer solution and ultra-purified water were added into ampules containing pyrene, obtaining serial solutions of various micelle concentrations ranging from 1.0×10^{-5} to 0.5 mg/mL. Fluorescence spectra were recorded using a spectrofluorophotometer with 373-nm excitation wavelength and 5-nm slit width. The release rate of PTX or RAP from micelles was investigated by a dialysis method. Briefly, the mixture of 0.5 mL micellar solution and 0.5 mL RPMI-1640 containing 10% FBS was dialyzed (molecular weight cut off = 14,000 Da) against 50.0 mL release medium (1 mol/L sodium salicylate) at 37 °C with gentle shaking at 100 rpm. Aliquots of release medium were withdrawn at predesigned time points (1, 2, 4, 6, 8, 12 and 24 h) and an equal volume of fresh release medium was added. The amount of released drug was quantified by HPLC method. The leakage of C6 from micelles was determined according to the procedures of release test, excepting that the release medium was replaced by RPMI-1640 containing 10% FBS.

1.4. Immunofluorescence for transferrin receptor

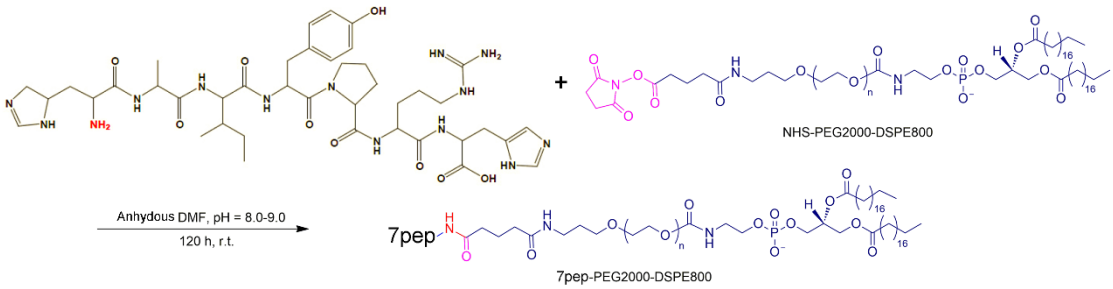
TfR expression on MCF-7 cells was determined by flow cytometry. MCF-7 cells were seeded in 12-well plates and incubated for 24 h at 37 °C. Then cells were washed and trypsinized to obtain the single cell suspension. TfR on plasma membrane was stained by incubating with 100 μL of PE mouse antihuman CD71 antibody for 20 min at room temperature. Negative control cells were incubated with an isotype control (PE mouse IgG2a) instead of anti-CD71 antibody. After washing with PBS, the PE fluorescence was measured by a flow cytometer (FACScan, Becton Dickinson, San Jose, CA) with 10,000 events collected.

2. Tables

Table S1 Resist diluting test of various drug-loaded micelles (mean ± SD, *n* = 3).

Formulations	Dilution	Size (nm)	PDI	EE (%)
M-PTX	1	15.91±1.94	0.134±0.066	93.2±5.4
	10	18.79±2.11	0.192±0.059	91.5±3.6
	100	19.20±2.36	0.183±0.072	88.7±5.7
7pep-M-PTX	1	16.70±1.34	0.104±0.085	90.1±2.3
	10	15.30±2.02	0.157±0.062	89.9±3.1
	100	17.72±3.15	0.172±0.079	86.3±5.9
M-RAP	1	15.31±1.11	0.109±0.099	94.0±4.2
	10	17.95±1.82	0.184±0.083	92.7±2.6
	100	18.84±2.56	0.191±0.075	89.4±4.8
7pep-M-RAP	1	15.55±1.48	0.150±0.065	95.1±3.3
	10	15.99±2.37	0.163±0.077	92.5±3.7
	100	17.26±2.09	0.178±0.091	87.4±6.0

3. Figures



Scheme S1 Synthesis scheme for conjugation of 7pep to NHS-PEG-DSPE.

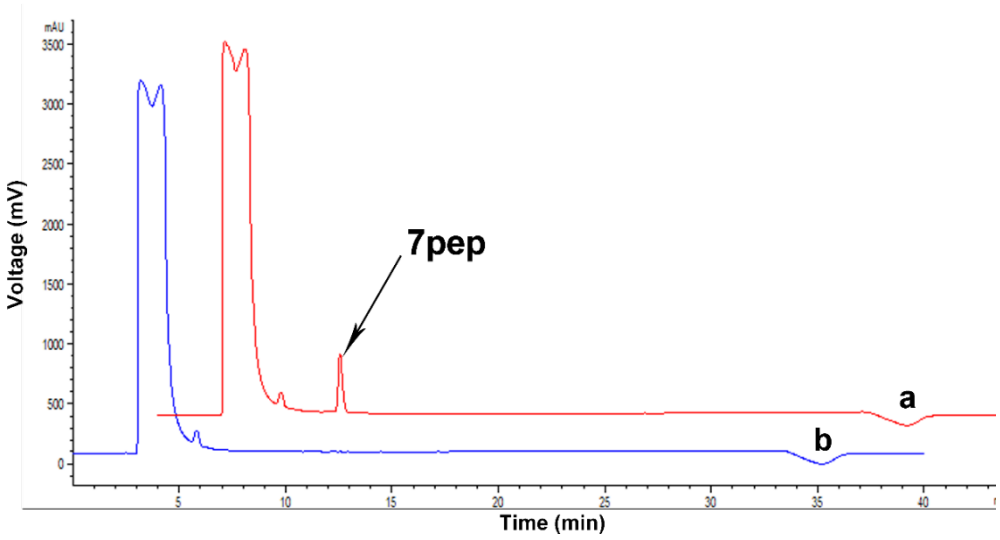


Figure S1 RP-HPLC profiles of unconjugated 7pep in the conjugation of 7pep-PEG-DSPE: free 7pep in reaction mixture (a) at the initial time and (b) after reaction for 120 h.

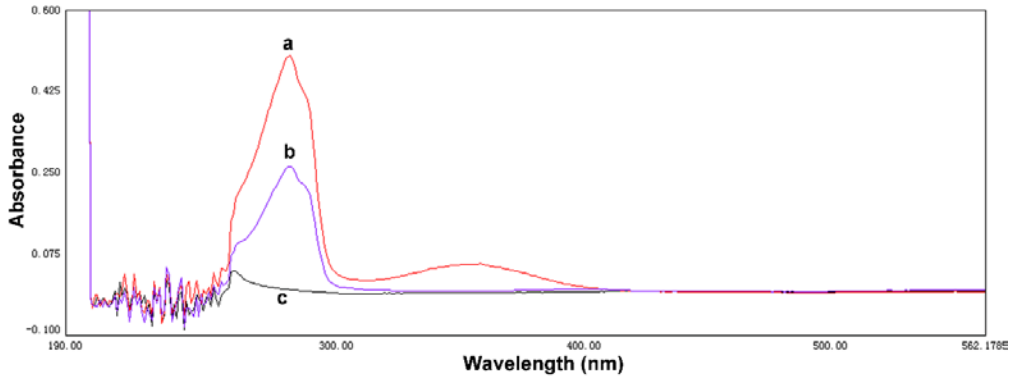


Figure S2 UV-Vis spectra scanning profiles of (a) 7pep, (b)7pep-PEG-DSPE and (c) NHS-PEG-DSPE in DMSO.

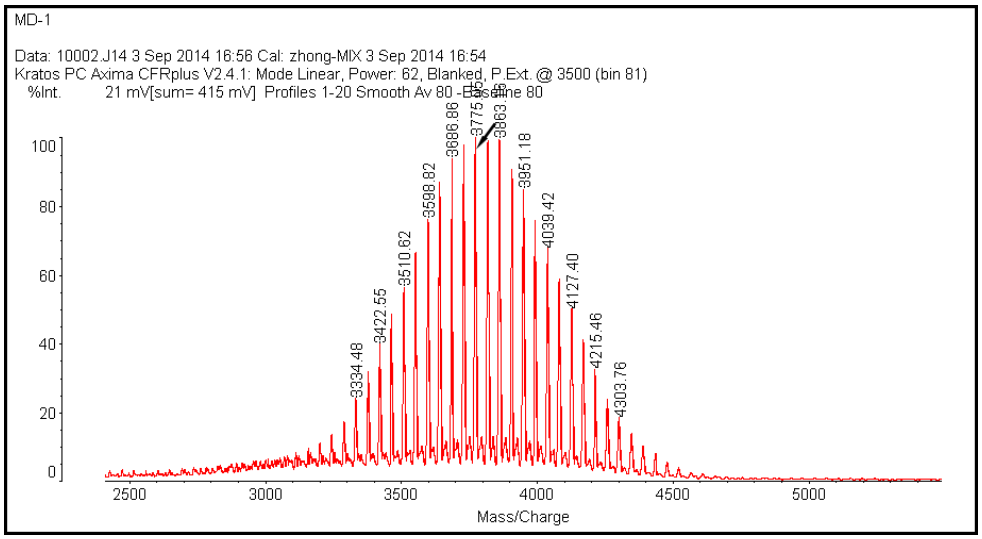


Figure S3 MALDI-TOF MS spectrum of 7pep-PEG-DSPE with a peak at the m/z range around 3775.6 Da.

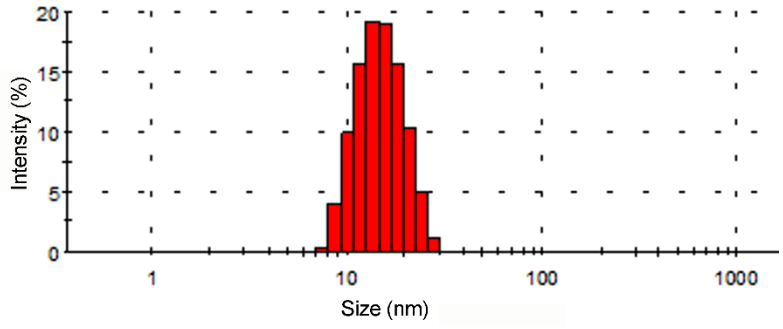


Figure S4 Particle size distribution of 7pep-M-PTX by DLS.

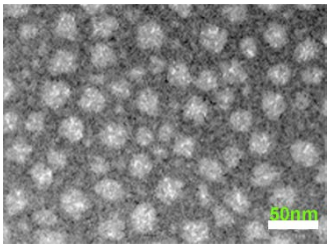


Figure S5 Morphology of 7pep-M-PTX by TEM.

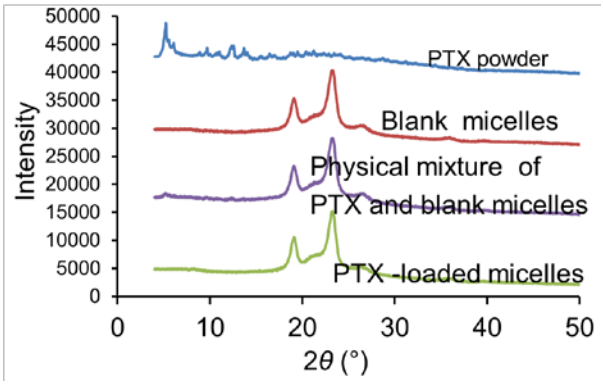


Figure S6 Powder X-ray diffraction patterns of various PTX preparations, including PTX powder, blank PEG-DSPE micelles, physical mixture of PTX plus blank micelles, and lyophilized PTX-loaded micelles.

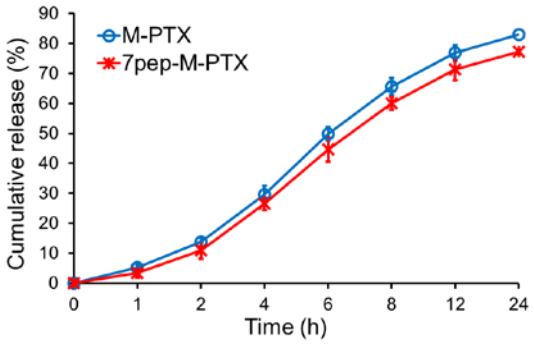


Figure S7 In vitro release of PTX from micelles in 1.0 mol/L sodium salicylate at 37 °C (mean ± SD, *n* = 3).

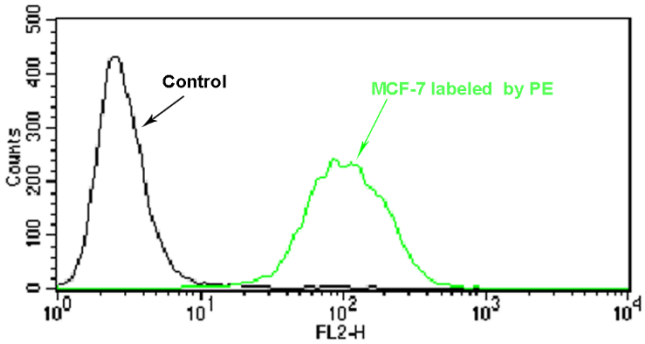


Figure S8 Characterization of TfR expression in MCF-7 cells.

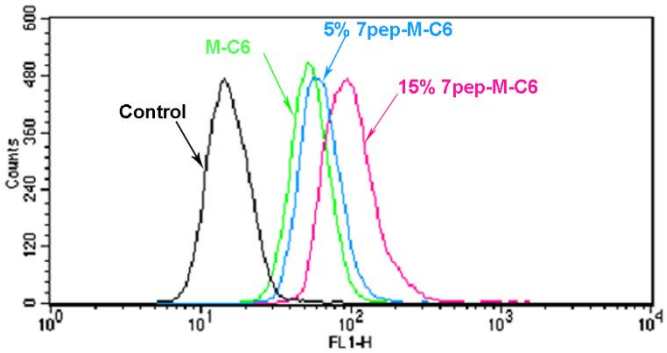


Figure S9 Optimization of 7pep density on micelles. MCF-7 cells were incubated with C6-loaded micelles with different ligand density (0%, 5%, 15%), and then analyzed by flow cytometry.

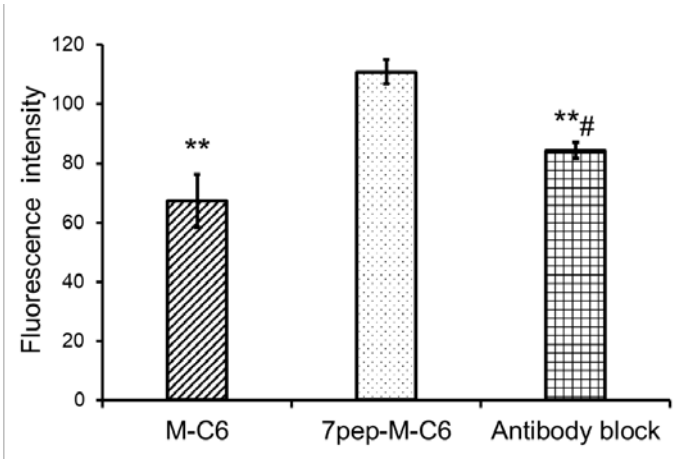


Figure S10 Quantitative analysis of competitive cellular uptake of various C6 formulations based on flow cytometric plots. Each bar represents mean fluorescence intensity ± SD (n = 3). $^{**}P < 0.01$ *vs* 7pep-M-C6; $^{\#}P < 0.05$ *vs* M-C6.

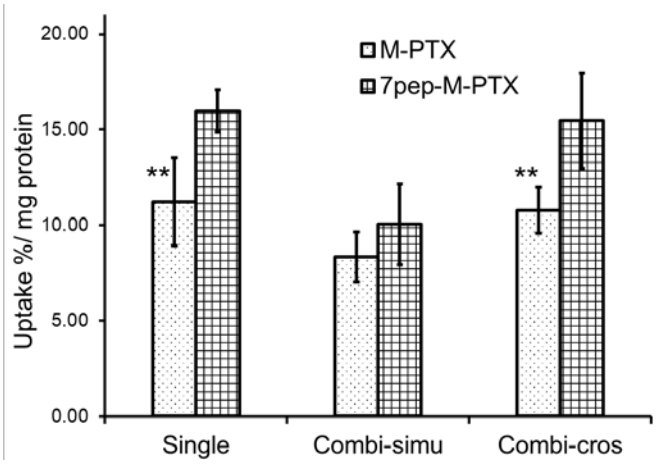


Figure S11 Cellular uptake of various PTX-loaded nanomedicines determined by HPLC. MCF-7 cells were incubated with M-PTX or 7pep-M-PTX in single or combination with RAP-loaded nanomedicines at a final concentration of 10 μmol/L for 2 h at 37 °C (mean ± SD, n = 3). For combination use, RAP-loaded nanomedicines and PTX-loaded micelles were added to the cells at the same time (Combi-simu), or RAP-loaded nanomedicines were added to the cells 6 h prior to the PTX-loaded micelles (Combi-cros). $^{**}P < 0.01$, M-PTX *vs* 7pep-M-PTX used in single or in Combi-cros.

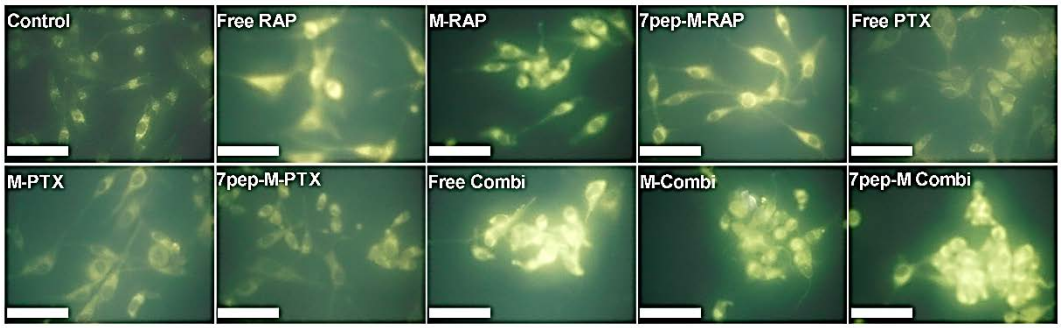


Figure S12 Autophagic vesicles stained by MDC. Cells were treated with Free RAP, M-RAP, 7pep-M-RAP, Free PTX, M-PTX, 7pep-M-PTX, Free Combi, PM-Combi and 7pep-M-Combi at a final concentration of 10 nmol/L for PTX and 100 nmol/L for RAP. Untreated cells were shown as Control. White scale bars = 50 μm.

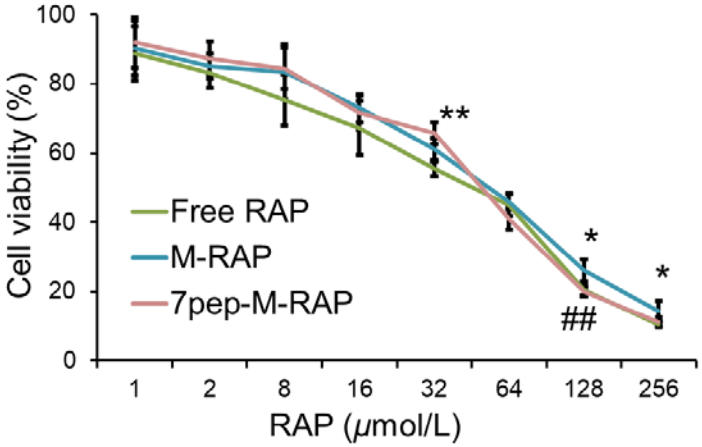


Figure S13 Cytotoxicity of various RAP formulations against MCF-7 cells for 48h by SRB assay (mean ± SD, n = 6). $^{*}P < 0.05$, M-RAP *vs* Free RAP; $^{**}P < 0.01$, 7pep-M-RAP *vs* Free RAP; $^{\#\#}P < 0.01$, M-RAP *vs* 7pep-M-RAP.

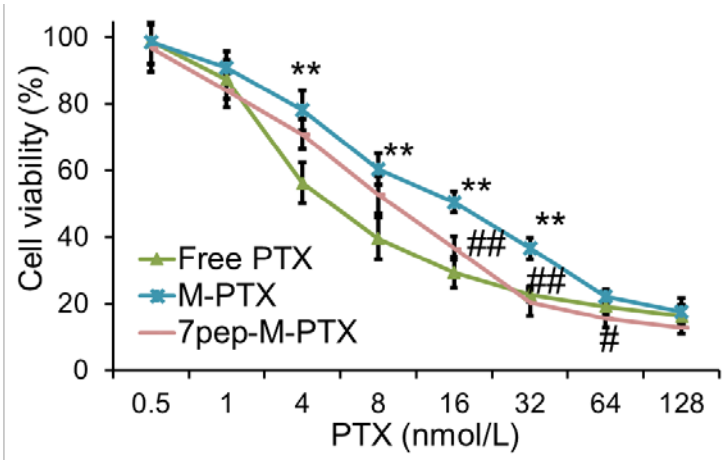


Figure S14 Cytotoxicity of various PTX formulations against MCF-7 cells for 48h (mean ± SD, n = 6). $^{*}P < 0.05$ and $^{**}P < 0.01$, M-PTX *vs* Free PTX; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, M-PTX *vs* 7pep-M-PTX.

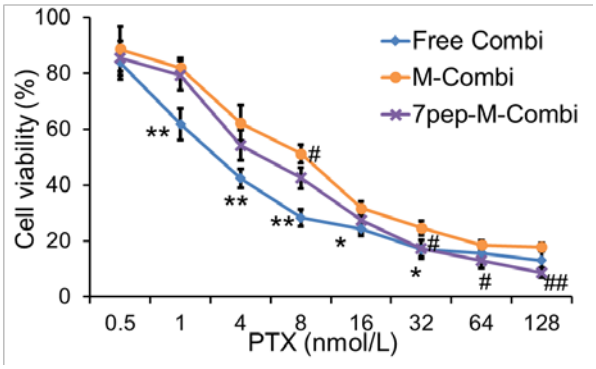


Figure S15 Comparison of the three combination groups (mean ± SD, n = 6). $^{*}P$ < 0.05 and $^{**}P$ < 0.01, Free Combi *vs* M-Combi; $^{\#}P$ < 0.05 and $^{\#\#}P$ < 0.01, M-Combi *vs* 7pep-M-Combi.

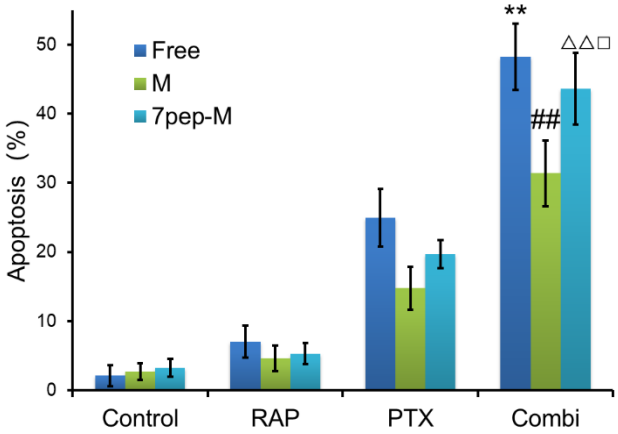


Figure S16 Quantitative analysis of in vitro cell apoptosis based on flow cytometric plots. Each bar represents the sum of early apoptotic cells and late apoptotic cells (mean ± SD, n = 3). $^{\star}P$ < 0.05 *vs* M-PTX; $^{**}P$ < 0.01 *vs* Free PTX; $^{\#\#}P$ < 0.01 *vs* M-PTX; $^{\triangle\triangle}P$ < 0.01 *vs* 7pep-M-PTX; $^{\square}P$ < 0.01 *vs* M-Combi.

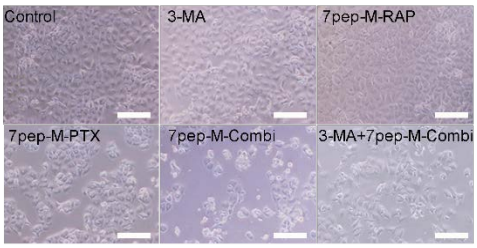


Figure S17 Typically morphological change of MCF-7 cells after treated with 3-MA, 7pep-M-RAP, 7pep-M-PTX, 7pep-M-Combi, 3-MA plus 7pep-M for 48 h, respectively. White scale bars = 100 μm.

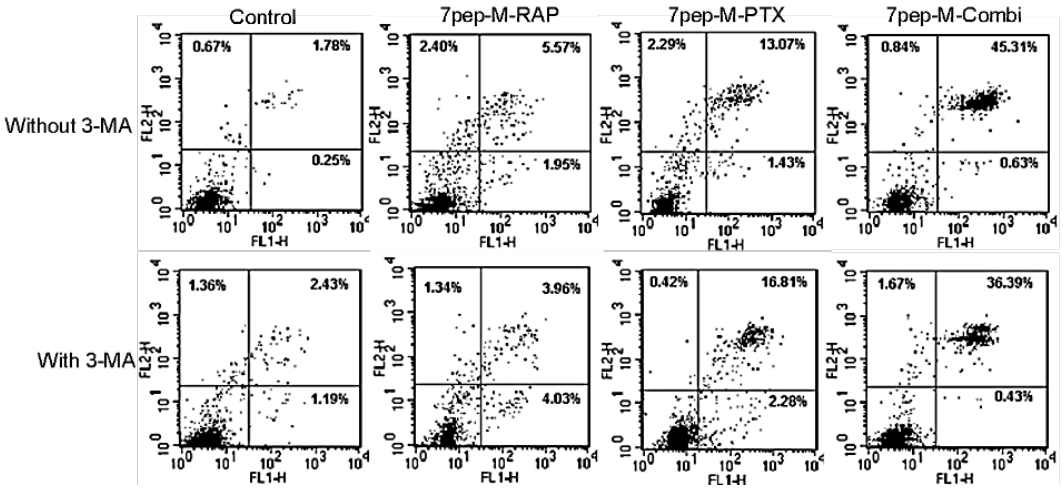


Figure S18 Effect of 3-MA on the in vitro cell apoptosis induced by 7pep-M-PTX in single or in combination with 7pep-M-RAP. The final concentration of PTX and RAP was 10 and 100 nmol/L, respectively. Double parameter dot plots show FITC-fluorescence (x axis) versus PI-fluorescence (y axis). Quadrants: lower left, normal live cells (annexin V^-/PI^-); lower right, early apoptotic cell (annexin V^+/PI^-); upper right, late apoptotic or necrotic cells (annexin V^+/PI^+); and upper left, mechanically injured cells (annexin V^-/PI^+).

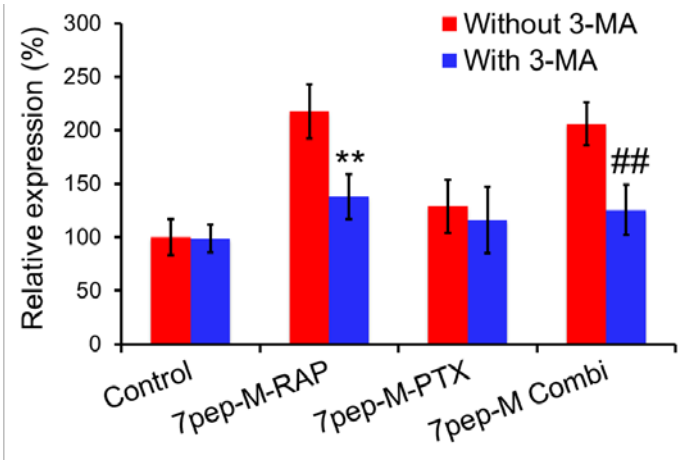


Figure S19 The inhibition effect of 3-MA on the LC3B expression measured by ELISA (mean ± SD, n = 3). $^{**}P < 0.01$ *vs* 7pep-M-RAP without 3-MA; $^{\#\#}P < 0.01$ *vs* 7pep-M-Combi without 3-MA.

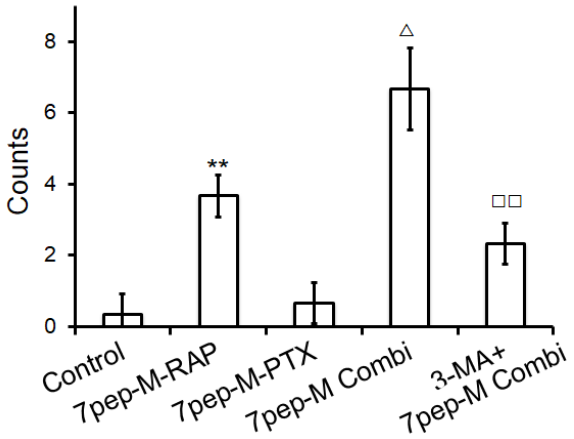


Figure S20 Quantitative analysis of autophagic vesicles in MCF-7 cells observed by TEM. Each bar on the histogram represents average count obtained from 3 randomly selected visual fields (mean ± SD, n = 3). $^{**}P < 0.01$ *vs* Control; $^{\triangle}P < 0.05$ *vs* 7pep-M-RAP; $^{\square\square}P < 0.01$ *vs* 3-MA+7pep-M Combi.

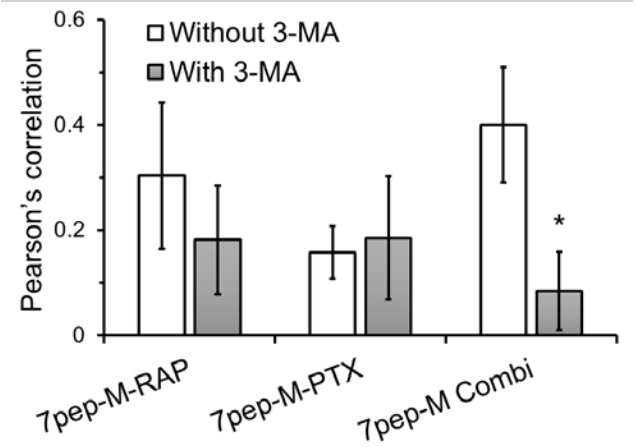


Figure S21 Quantitative colocalization analysis of Cyto-ID specifically labeled autophagic vesicles and Mitotracker labeled mitochondria in MCF-7 cells treated without or with 3-MA (mean ± SD, n = 6). $^{*}P < 0.05$ *vs* 7pep-M Combi without 3-MA.

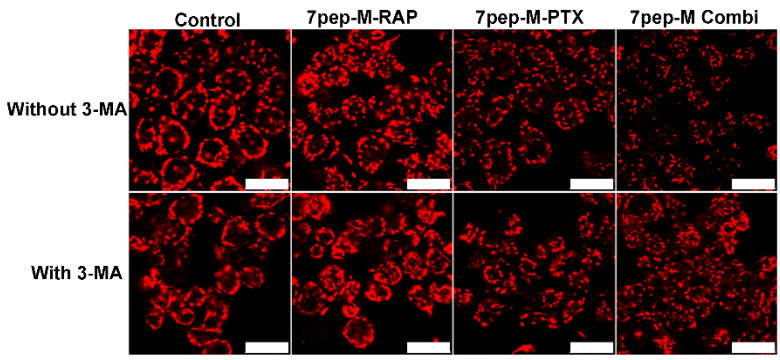


Figure S22 Decline of mitochondria membrane potential ($\Delta\psi$) measured by confocal microscopy. White scale bars = 25 μm.

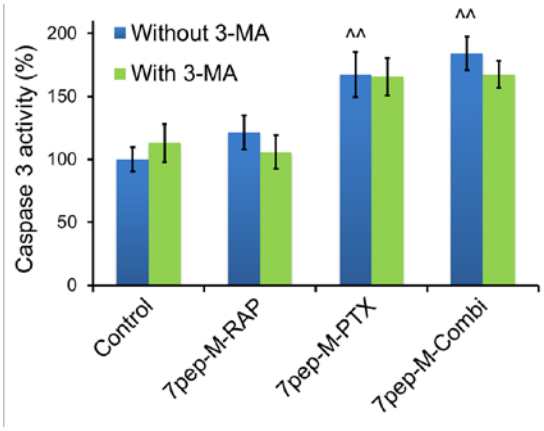


Figure S23 Activity of caspase-3 in MCF-7 cells induced by various formulations (mean ± SD, n = 3). ^^P < 0.01 *vs* Control.

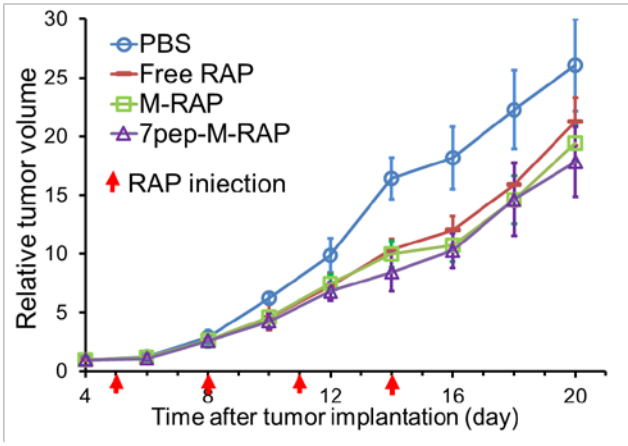


Figure S24 Tumor growth curves of mice treated with various RAP formulations. Red arrows indicate the time for RAP injection after tumor cells inoculation (mean ± SD, n

= 6).

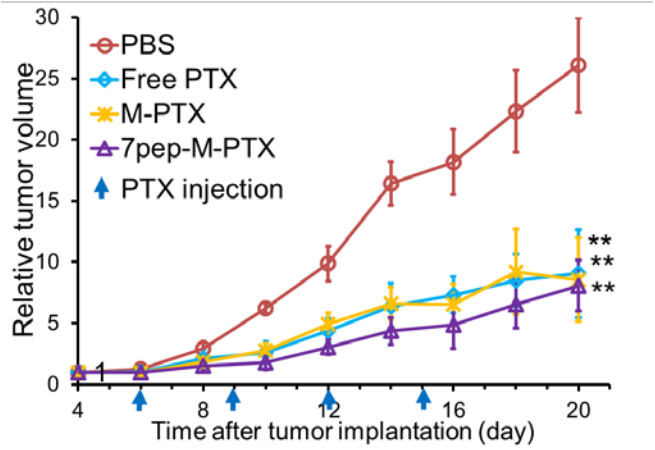


Figure S25 Tumor growth curves of mice administrated with various PTX formulations. Blue arrows indicate the time for injection (mean ± SD, n = 6). $^{**}P$ < 0.01 *vs* PBS.

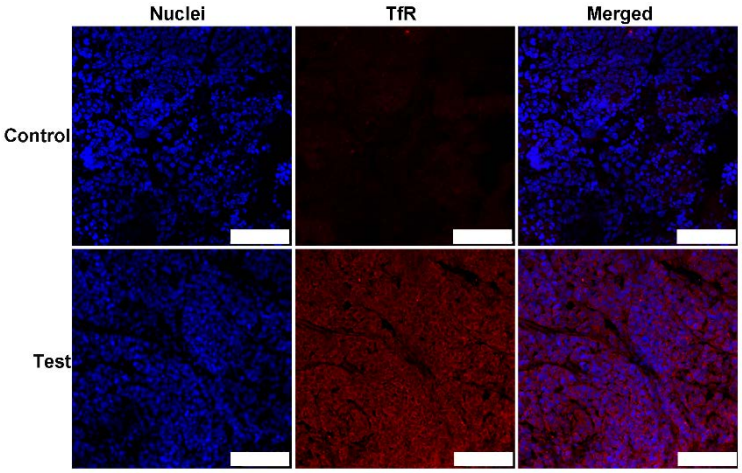


Figure S26 The TfR expression on MCF-7 tumor tissue determined by immunofluorescence staining. White scale bars = 100 μm.

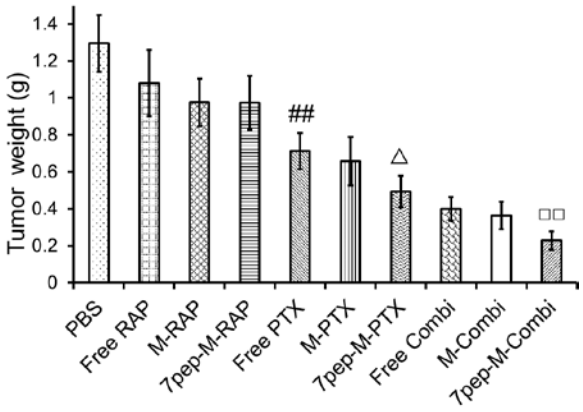


Figure S27 Weight of the excised tumor masses from different treatment groups (mean ± SD, $n = 6$). $^{\#\#}P < 0.01$ *vs* 7pep-M-RAP; $^{\triangle}P < 0.05$ *vs* M-PTX; $^{\square\square}P < 0.01$ *vs* M-Combi.

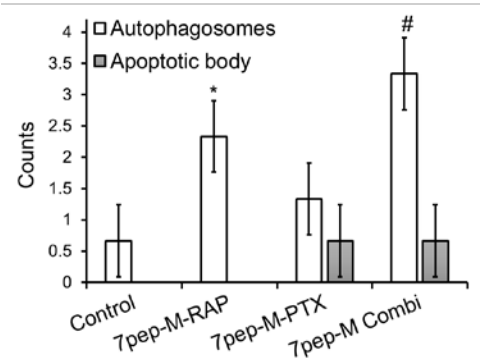


Figure S28 Quantitative analysis of autophagic vesicles and apoptotic body in MCF-7 excised tumor tissues observed by TEM. Each bar on the histogram represents average count obtained from 3 randomly selected visual fields (mean ± SD, $n = 3$). $^{*}P < 0.05$ *vs* autophagosomes in Control; $^{\#}P < 0.05$ *vs* autophagosomes in 7pep-M-PTX group.

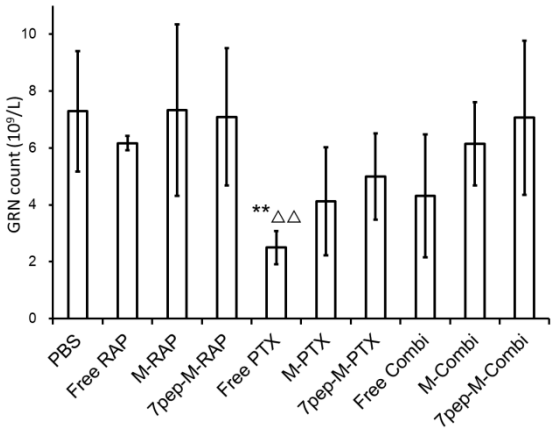


Figure S29 Effects of different formulations on neutrophilic granulocyte (GRN) counts at the end of test. Each bar represents mean ± SD (n = 5). $^{**}P < 0.01$ *vs* PBS; $^{\triangle\triangle}P < 0.01$ *vs* 7pep-M-PTX.

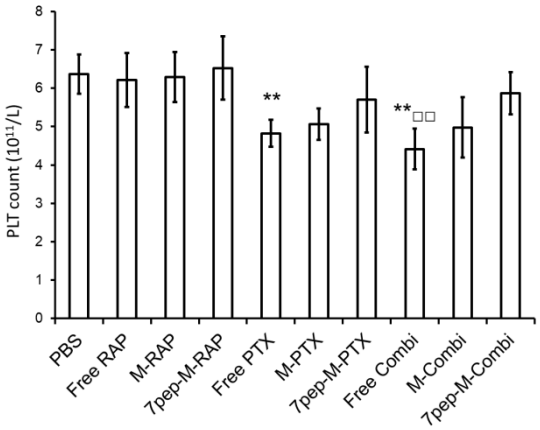


Figure S30 Effects of different formulations on platelet (PLT) counts at the end of test. Each bar represents mean ± SD (n = 5). $^{**}P < 0.01$ *vs* PBS; $^{\square\square}P < 0.01$ *vs* 7pep-M-Combi.

References

1. Song X, Li R, Deng H, Li Y, Cui Y, Zhang H, et al. Receptor mediated transcytosis in biological barrier: The influence of receptor character and their ligand density on the transmembrane pathway of active-targeting nanocarriers. *Biomaterials* 2018;180:78-90.

2. Yu C, He B, Xiong M, Zhang H, Yuan L, Ma L, et al. The effect of hydrophilic and hydrophobic structure of amphiphilic polymeric micelles on their transport in epithelial MDCK cells. *Biomaterials* 2013;34:6284-98.