Electronic Supplementary Information (ESI)

A peptide-based pH-sensitive drug delivery system for targeted ablation of cancer cells

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1. HPLC analysis of AP2H-hydrazide

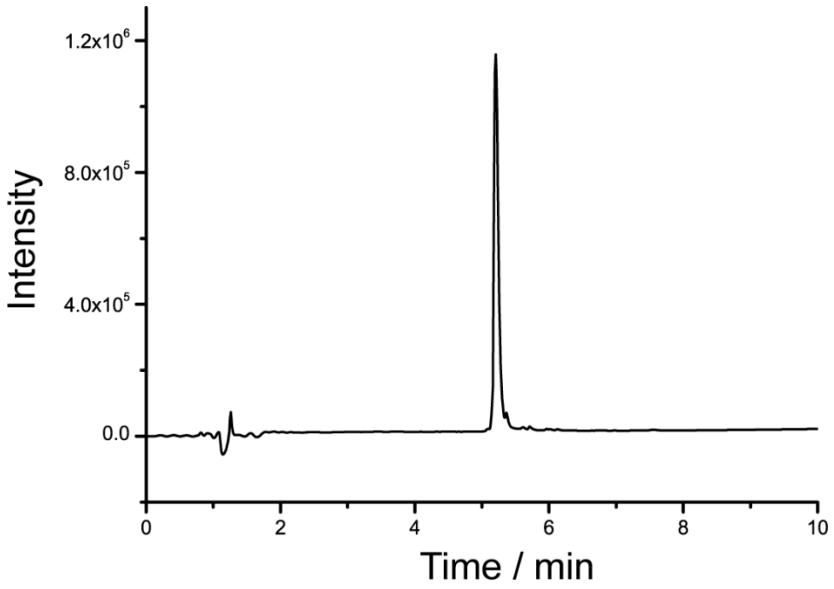


Figure S1. HPLC analysis of AP2H-hydrazide on a C_{18} column. Gradient: 0-10min, 10%B–50%B (A: 0.1% TFA/H_2O, B: 0.1% TFA/CH_3CN).

2. ESI-IT MS characterization of AP2H-hydrazide

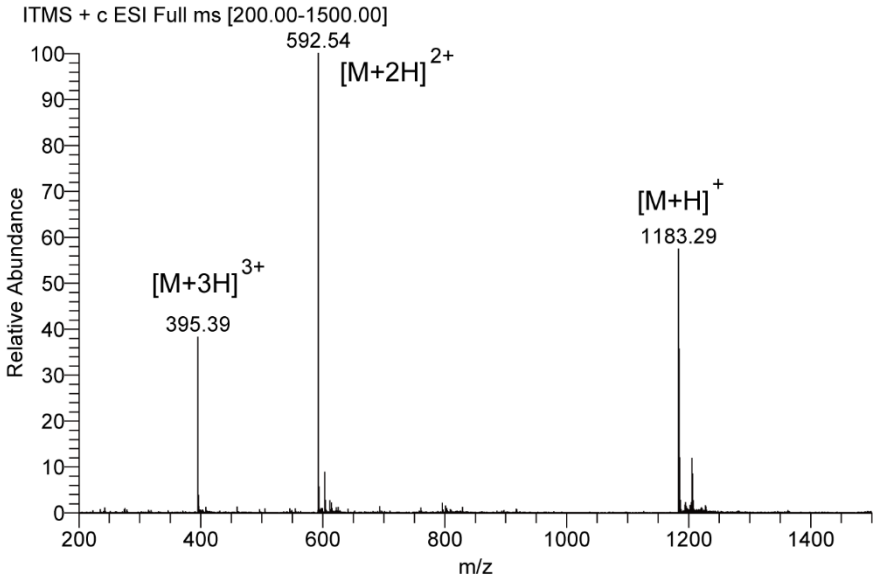


Figure S2. ESI-IT mass spectrum of AP2H-hydrazide (Positive ion mode). m/z $[M+2H]^{2+}$ calcd: 592.32; found: 592.54.

3. HPLC analysis of AP2H-hydrazone-DOX

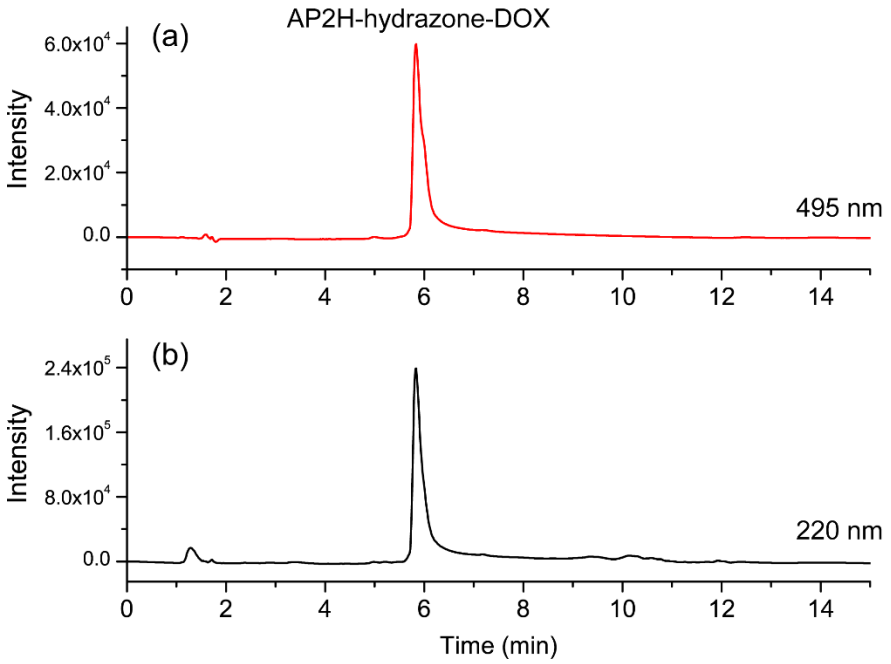


Figure S3. HPLC analysis of AP2H-hydrazone-DOX on a C_{18} column monitored at the wavelength of (a) 495 nm and (b) 220 nm. Gradient: 0-10-10.01-15 min, 40%B–70%B–90%B-90%B (A: H2O containing 10 mM NH_4OAc, B: acetonitrile).

4. ESI-IT MS characterization of AP2H-hydrazone-DOX

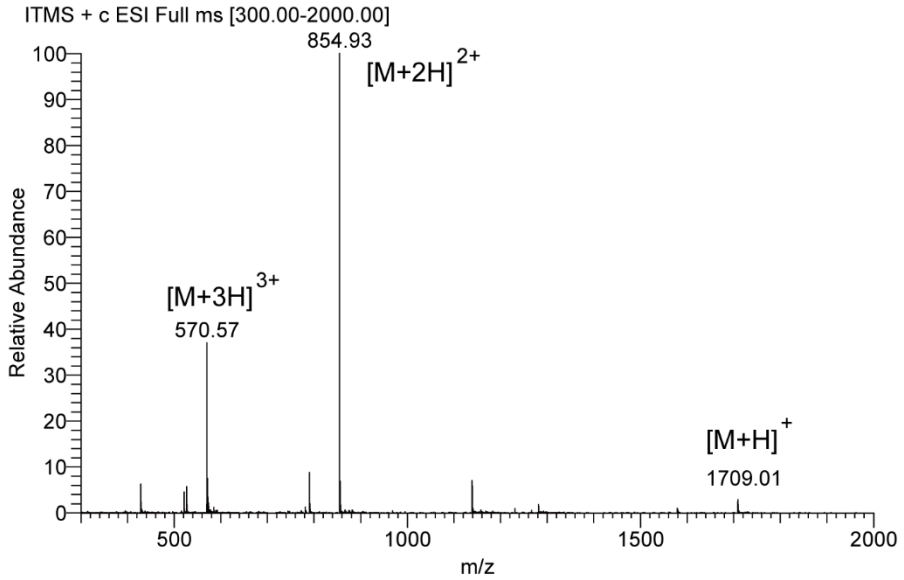
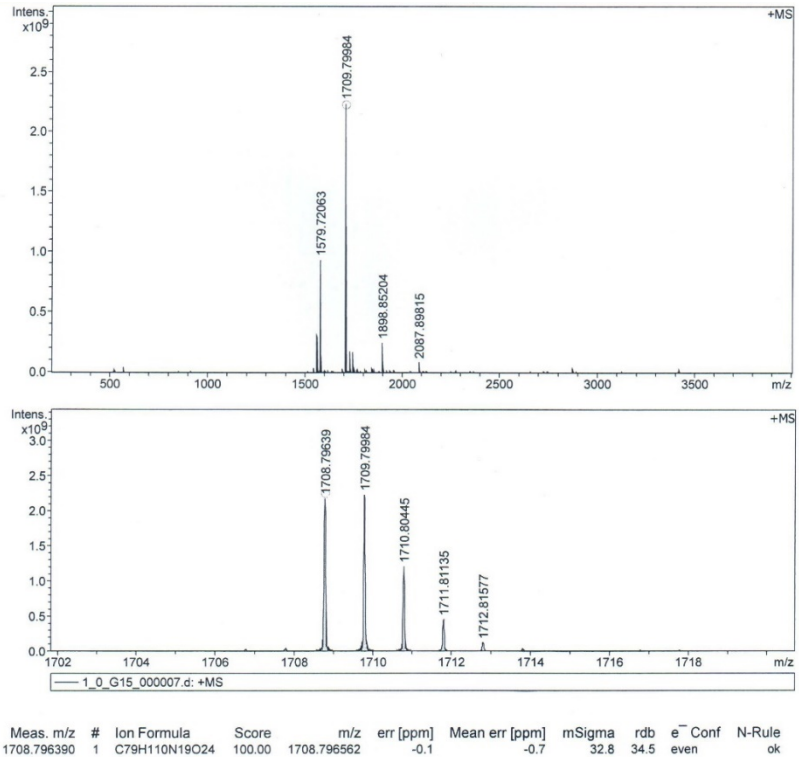


Figure S4. ESI-IT mass spectrum of AP2H-hydrazone-DOX (Positive ion mode). m/z $[M+2H]^{2+}$ calcd: 854.93; found: 854.90.

5. High resolution MALDI-TOF MS characterization of AP2H-hydrazone-DOX



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	Mean err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
1708.796390	1	C79H110N19O24	100.00	1708.796562	-0.1	-0.7	32.8	34.5	even	ok

Figure S5. High resolution MALDI-TOF mass spectrum of AP2H-hydrazone-DOX operated in positive ion mode: m/z $[M+H]^+$ calcd: 1708.7966; found: 1708.7964.

6. HPLC-ESI-IT MS analysis of AP2H-hydrazone-DOX after acidic catalyzed hydrolysis

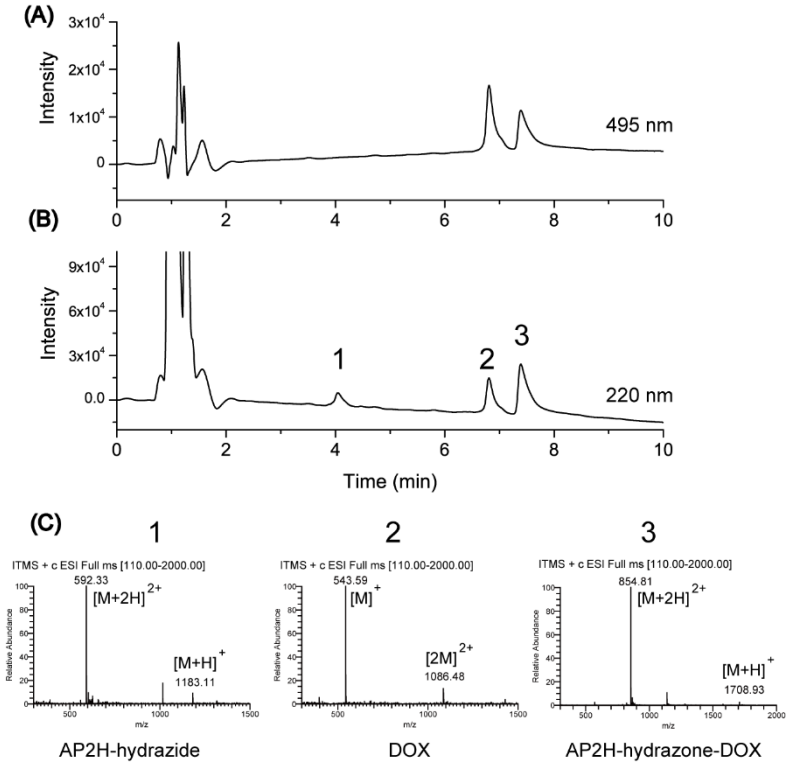


Figure S6. HPLC-ESI-IT MS analysis of AP2H-hydrazone-DOX after acidic catalyzed hydrolysis (pH 5.0, 16 h). Reaction solutions were monitored at wavelengths of (a) 495 nm and (b) 220 nm. (c) ESI-IT mass spectrum of AP2H-hydrazide, DOX and AP2H-hydrazone-DOX.

7. Experimental Section

Materials and Apparatus

9-Fluorenylmethoxycarbonyl (FMOC)-protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-Hydroxybenzotriazole•H_2O (HOBT) were purchased from Siam (Chicago, IL). FMOC-amino acid-Wang resin was from Advanced ChemTech (Louisville, KY). Acetonitrile (ACN), methanol (MeOH), trifluoroacetic acid (TFA), triisoproyl silane (TIS), FMOC-hydrazide and doxorubicin (DOX) were purchased from Fisher Scientific or Sigma-Aldrich. Dimethylformamide (DMF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), piperidine, 4-methylmorpholine (NMM), diethyl ether and succine anhydride (SA) were purchased from Beijing Chemical Works. Sodium acetate and ammonium acetate were purchased from Beijing Chemical Reagents Company. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from

Invitrogen (California). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. Hoechst 33342 was obtained from Solarbio (Beijing, China). LysoTracker Green DND-26 was from life technologies (Thermo Fisher Scientific). Ultrapure water from a Milli Q water purification system (Millipore, Bedford, MA) was used throughout. Other materials used for synthesis are commercially available and used as received. All solvents were purified and dried following standard procedures unless otherwise stated.

The HPLC–ESI–IT–MS analysis was performed on an UltiMate 3000 UHPLC system equipped with a diode-array detector and an LCQ Fleet mass spectrometer (ThermoFisher, San Jose, CA, USA). The chromatographic column was a ThermoFisher Hypersil-gold C_{18} column with 3 μm particle diameter (column dimensions 100×2.1 mm).

Synthesis of hydrazide linker-functionalised AP2H peptide

Synthesis of AP2H-hydrazide was performed by standard FMOC solid phase peptide synthesis (Scheme 1). Fmoc-Gly-Wang resin (Gly loading: 0.4 mmol/g) was used as the starting material. After removal of Fmoc-group with 20% (v/v) piperidine in DMF, a 3-fold excess of Fmoc-amino acid was added using HBTU/NMM as coupling condition. The completion of each coupling step was verified by Kaiser Test. After the last amino acid (Ile) was coupled to the resin and followed by deprotection of FMOC-group, 3-fold excess of succine anhydride (SA) was added using NMM as a base catalyst. 3-fold excess of FMOC-hydrazide, HBTU and HOBT were added sequentially for the reaction with the AP2H loaded resin in DMF at room temperature overnight. After removal of the FMOC-group, the resin was washed with DMF, DCM, MeOH and dried in a vacuum oven. Subsequently, the resin was incubated with a freshly prepared TFA cocktail (95%TFA, 2.5%H_2O and 2.5% TIS as scavengers) for 2h and filtered. The filtrate was vacuum-dried with rotary evaporation and the residue was treated with cold diethyl ether. The white precipitate was collected by centrifugation and washed with cold diethyl ether three times. The product was dried and purified on a semi-preparative HPLC column (Dikma-C_{18}, 250 mm×10 mm i.d.) using water with 0.1% TFA as the aqueous phase and ACN with 0.1% TFA as the organic phase.

Synthesis of AP2H-hydrazone-DOX

AP2H-hydrazide (11.8 mg, 10 μmol), doxorubicin hydrochloride (11.6 mg, 20 μmol), anhydrous methanol (3 mL) and trifluoroacetic acid (6 μL) were added to a 5

mL sealed vial (Scheme 1). After being stirred in the dark at room temperature for 24 h, the reaction mixture was purified with a semi-preparative HPLC column using a 28%-55% gradient elution system with aq. NH_4OAc buffer (10 mM, pH 7.0) (solvent A) and MeCN (solvent B) over 20 min at 2.0 mL/min. The final product AP2H-hydrazone-DOX, was obtained as red powder after lyophilization.

DOX Release Kinetics

The drug release profiles of AP2H-hydrazone-DOX were assessed under physiological (PBS, pH 7.4) and acidic conditions (PBS, pH 6.0 and 5.0), mimicking the tumor microenvironment (~pH 6.0) and endo-lysosomal environment (~pH 5.0). In a typical experiment, AP2H-hydrazone-DOX was dissolved in buffer to obtain a final concentration of 10 μM. The mixed solutions were then shaken at a constant speed (120 rpm) at 37 ^oC. At different time intervals (0, 1, 2, 4, 8, 12, 24, 36 and 48h), 100 μL of the solution was taken out for HPLC analysis (triplicate, 30 μL each time). The RP-HPLC-IT-MS system with a thermo Hypersil-Gold C_{18} column was employed. Gradient: 0-10 min, 15%B-40%B; solvent A: NH_4OAc buffer (20 mM, pH 7.0), solvent B: MeCN; flow rate: 0.3 mL/min. The diode-array detector was set at a wavelength of 220 and 495 nm. MS spray voltage was 4.5 kV for positive ion mode. The capillary temperature was 300 ^oC.

Cells

A549 cells, HepG2 cells and HEK293 cells were from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured at 37 ^oC and 5% CO_2 in high-glucose DMEM supplemented with 10% fetal bovine serum (Hyclone). The HEK293 cell line was used in the control experiments.

Cellular uptake observed by confocal laser scanning microscopy (CLSM)

In 35 mm glass-bottomed dishes, the cells (approximately 1.0×10^5 mL^{-1}) were seeded and cultured overnight for adhesion. Then the cells were treated with AP2H-hydrazone-DOX solution or DOX solution (200 μL, 10 μM), respectively. After a certain period of incubation, the cells were carefully washed with PBS three times. Fluorescence imaging experiments were performed on an FV 1000-IX81 CLSM (Olympus, Japan). The objective used for imaging was a UPLSAPO 100× oil-immersion objective (Olympus). Image processing and analysis was performed on Olympus software (FV10-ASW). For costaining assays, the AP2H-hydrazone-DOX or DOX loaded cells were subjected to incubation with Hoechst 33342 solution for 20

min. After being washed with PBS, the cell samples were observed with CLSM. Hoechst 33342 was excited by a 50 mW, 405 nm Laser Head FV5-LD405-2 and collected with a band-pass filter within the range of 425−475 nm. The red-emission from AP2H-hydrazone-DOX or DOX was collected with a band-pass filter within the range of 550−650 nm. For costaining assay with lysosomal tracker, the AP2H-hydrazone-DOX or DOX loaded cells were incubated with LysoTracker Green solution (60 nM in PBS) at 37 ^{o}C for 2 hours. After replacing the dye solution with PBS, the cell samples were observed with CLMS. A FV5-LAMAR 488 nm laser was used as the excitation source. The green fluorescence from the lysosomal tracker was collected with a band-pass filter within the range of 500−550 nm, and the red emission from AP2H-hydrazone-DOX or DOX was collected with a band-pass filter within the range of 570−670 nm.

Cytotoxicity Assays

The cells were seeded in 96- well plates at a density of about 2000 cells/mL and cultured for 24 hours. Serial dilutions of AP2H-hydrazide, AP2H-hydrazone-DOX or DOX were prepared in cultured medium and added to the wells. The final concentrations of these compounds ranged from 0.01 to 10 μM. After incubation for 48 hours at 37 ^{o}C with 5% CO_2, cell viability was determined by MTT assay. The IC_{50} value for cytotoxicity was calculated from sigmoidal fits of the dose response curve of AP2H-hydrazone-DOX or DOX.

The viability of different cells was evaluated by the standard MTT assay. The culture medium was carefully removed, and 100 μL of freshly prepared MTT solution (0.5 mg/mL in culture medium) was added to each well. After incubation at 37 °C for 4 hours, the MTT solution was removed, and 100 μL of DMSO was added to dissolve the formazan crystals. The plate was shaken for 10 min to fully dissolve formazan and homogenize. Absorbance values of the wells were read with a microplate reader at 492 nm (BIO RAD, iMark). The cell viability rate (VR) was calculated from the following equation: $VR = A/A_0 \times 100\%$, where A is the absorbance of the experimental group and A_0 is the absorbance from the cells cultured in serum-supplemented medium without any treatment. All data were obtained from three repeatedly parallel experiments.

Statistical analysis was performed by IBM SPSS statistic 21 software, using one-way ANOVA with Turkey test. When P-values were 0.05 or less, differences were

considered statistically significant.