

## Supporting Information

### **Tandem Molecular Self-assembly of Drug-Peptide Conjugation Effectively Inhibits Lung Tumor Growth**

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## Chemicals and Materials

2-Chlorotriyl Chloride Resin (1.2 mmol/g) was purchased from Nankai University resin Co. Ltd. Fmoc-amino acids and o-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) were obtained from GL Biochem (Shanghai, China). Sodium Dithionite (SDT) was bought by J&K Co. Ltd. Alkali phosphatase (30 U/ $\mu$ L) was obtained from Takara (D2250, Dalian, China) Bio. Inc. Cy5.5 NHS ester (non-sulfonated) purchased from APEXBIO, RedDot™ 1 (200 $\times$ ) purchased from Biotium. Growth factor Matrigel purchased from BD Biosciences, FITC-CD31 purchased from BioLegende. Commercially available reagents and solvents were used without further purification, unless noted otherwise.

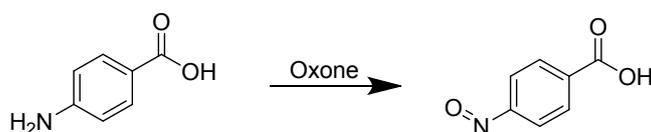
## General methods

The synthesized compounds were characterized by  $^1\text{H}$  NMR (Bruker ARX-400) using DMSO- $d_6$  as the solvent. HPLC was conducted at LUMTECH HPLC (Germany) system using a C18 RP column with methanol (0.05% of TFA) and water (0.05% of TFA) as the eluents. LC-MS was conducted at the LCMS-20AD (Shimadzu) system, the eluent buffer comprised of acetonitrile (0.035% of TFA) and water (0.035% of TFA). HR-MS was performed at the Agilent 6520 Q-TOF LC/MS using ESI-L low concentration tuning mix (Lot No. LB60116 from the Agilent Tech.). The morphology conversion of the peptide derivatives was measured by TEM performed on a Tecnai G2 F20 system, operating at 200 kV. Cellular uptake and drug tracking images were taken by a confocal laser scanning microscopy (Leica TSC SP5, Germany). Live animal imaging performed by IVIS Lumina II (Xenogen, USA).

## Synthesis and characterization

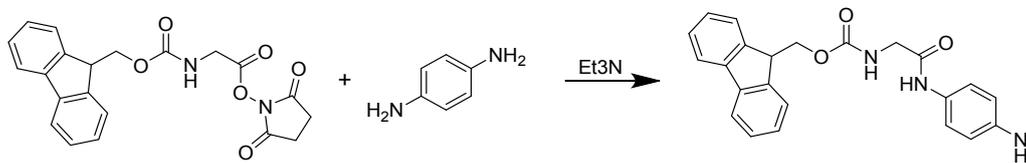
### Synthesis of Fmoc-azobenzene

According to previous study,<sup>1</sup> the synthesis procedure is following:

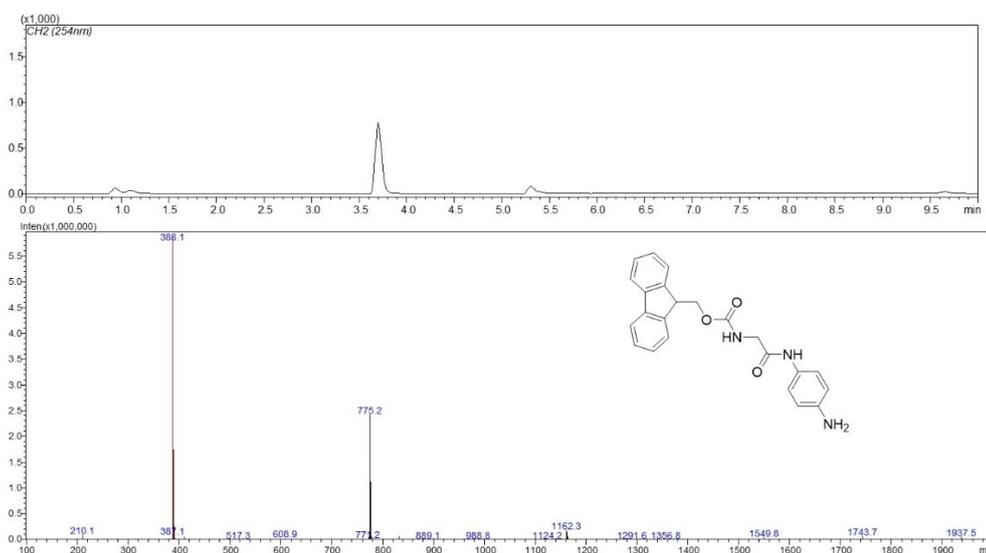


Firstly, to a suspension of 3-amino benzoic acid (10 mmol, 1.37 g) in DCM (50 mL), the solution of Oxone (20 mmol, 12.28 g) in water (100 mL) was added. The

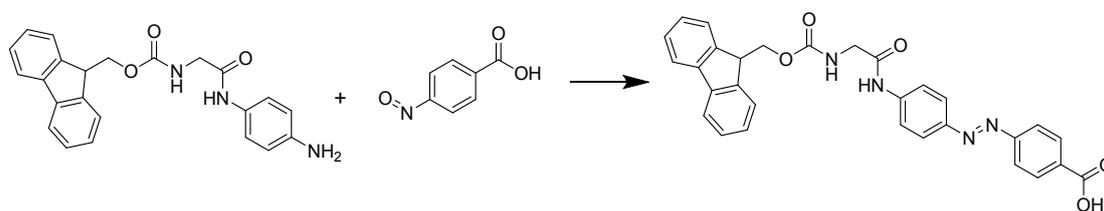
mixture was stirred at room temperature for 2 h. The precipitate was filtered and washed by water and ethanol three times respectively. The yellowish solid was obtained and directly used for next step.



To a solution of benzene-1,4-diamine (10 mmol, 1.37g) and Et<sub>3</sub>N (20 mmol, 1.6 mL), Fmoc-Gly-NHS (10 mmol, 3.94 g) was added. The mixture was stirred at room temperature overnight. The precipitate was filtered and washed by DCM and anhydrous ether three times. Purple solid product Comp.S1 was obtained with a yield of 65%, then used for next step



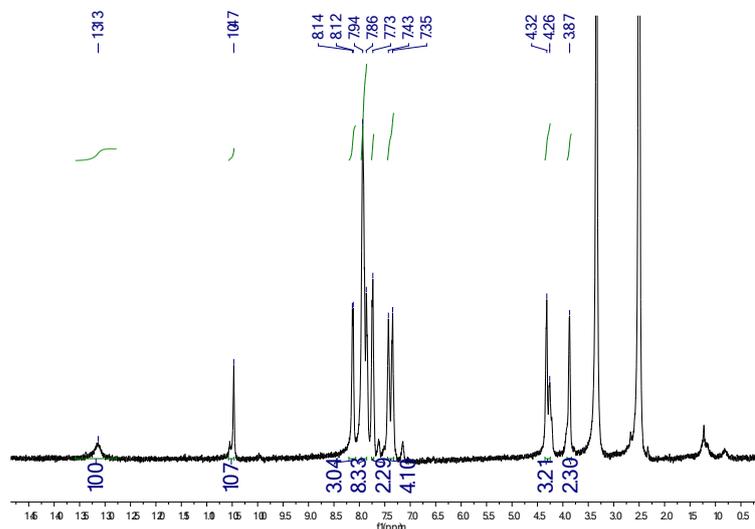
**Figure S1.** The mass spectrum of **Comp.S1**



**Scheme S1.** Synthetic route to **Comp. S2**.

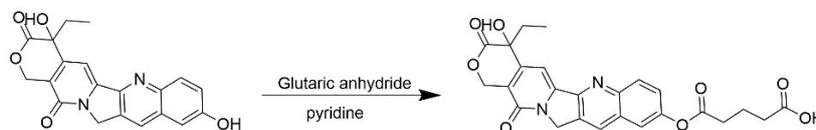
To a green solution of 3-nitrobenzoic acid (6 mmol, 906 mg) in DMSO/AcOH (1:1, 48 mL), Comp. S1 was added. The mixture was stirred at room temperature overnight. The reaction was monitored by LC-MS. 30 mL of water was added to quench reaction, and the precipitate was filtered and washed by water three times. The solid

product was obtained with a yield of 75% and directly used for next step.  $\delta_{\text{H}}$  (400 MHz, DMSO) 13.13 (1 H, s), 10.47 (1 H, s), 8.13 (3 H, d,  $J$  7.0), 8.01 – 7.63 (8 H, m), 7.39 (6 H, d,  $J$  33.5), 4.29 (3 H, d,  $J$  23.0), 3.87 (2 H, s).



**Figure S2.**  $^1\text{H}$ NMR spectrum of *Comp. S2*.

### synthesis of HCPT-Glutaric acid derivative



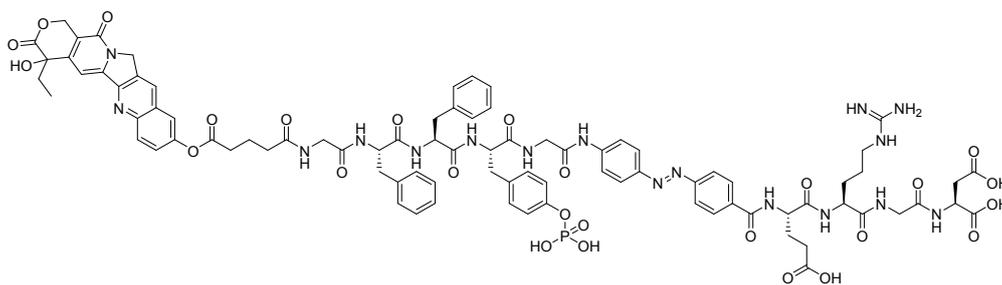
10-Hydroxycamptothecin (1.82g, 5mmol) and Glutaric anhydride (0.592g, 5.2mmol) were dissolved in pyridine (40mL), and the resulting solution was stirred at room temperature for 48h. The reaction was monitored by LC-MS. The solution was then removed under reduced pressure to obtain crude product, to obtain crude product re-dissolved in ethyl acetate (100ml), and then washed with 1M HCl (45ml) to get rid of pyridine for three times. The ethyl acetate was removed under vacuum and then add 10ml  $\text{H}_2\text{O}$  for freeze-drying to get crude product. The crude product used for SPPS as capping group to synthesis drug-peptide conjugation.

### Peptide Synthesis and Characterization

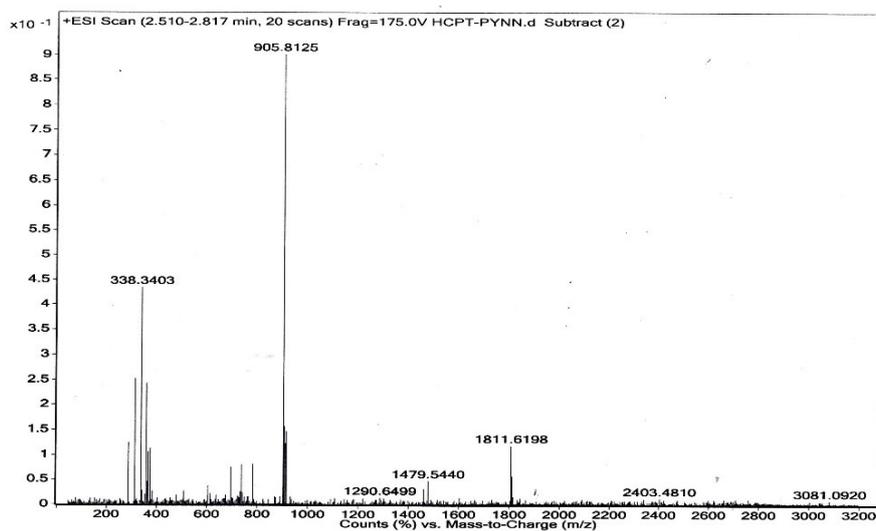
The peptide derivatives were prepared by solid phase peptide synthesis (SPPS) using 2-Chlorotrityl Chloride Resin and the corresponding N-Fmoc protected amino acids with side chains properly protected by a tertbutyl group (tBu) or 2,2,5,7,8-

Pentamethyl-chroman-6-sulfonyl (Pmc). 20% Piperidine DMF solution was used during deprotection of the Fmoc group. O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) was used as the coupling reagent. Fmoc-protected azobenzene group as amino acid can be used in SPPS. Finally, peptide derivative was cleaved using 95% of trifluoroacetic acid with 2.5% of PhSCH<sub>3</sub> and 2.5% of H<sub>2</sub>O for 30 min under dark. The crude products were purified by High performance liquid chromatography (HPLC) then frozen-dry to obtain pure products.

**Compound 1:**

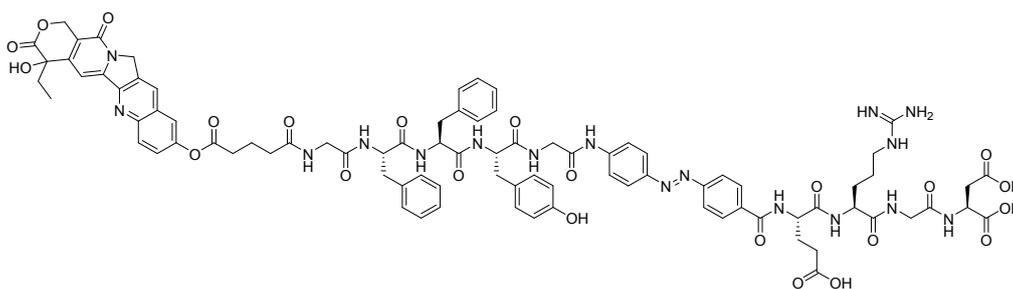


HR-MS: calc M<sup>+</sup>=1809.6137, obsvd (M+2H)<sup>2+</sup>=905.8125.

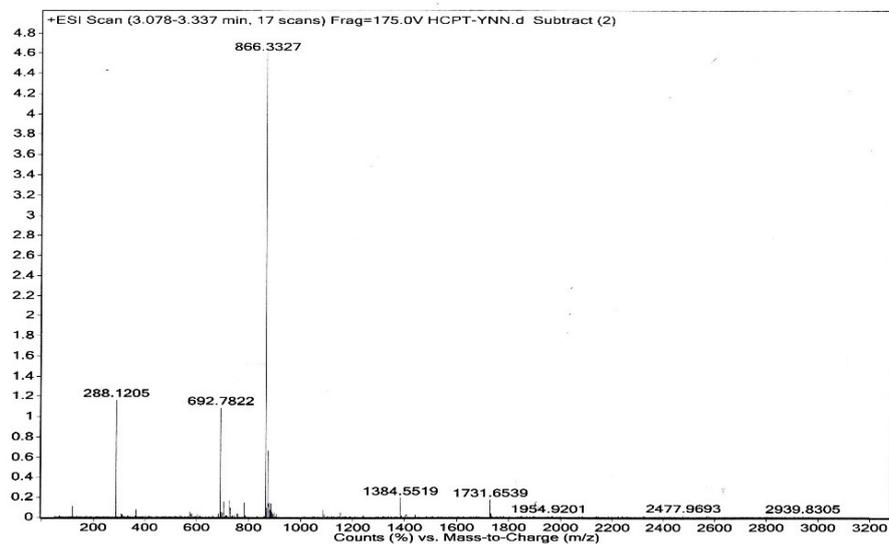


**Figure S3.** HR-MS spectrum of *Comp. 1*.

## Compound 2:

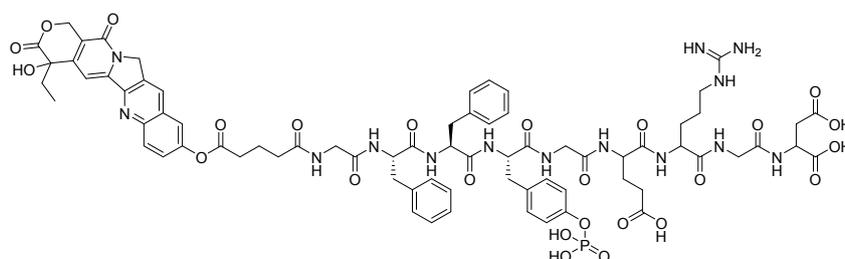


HR-MS: **Comp. 2**: calc  $M^+ = 1729.6494$ , obsvd  $(M+2H)^{2+} = 866.3327$ .

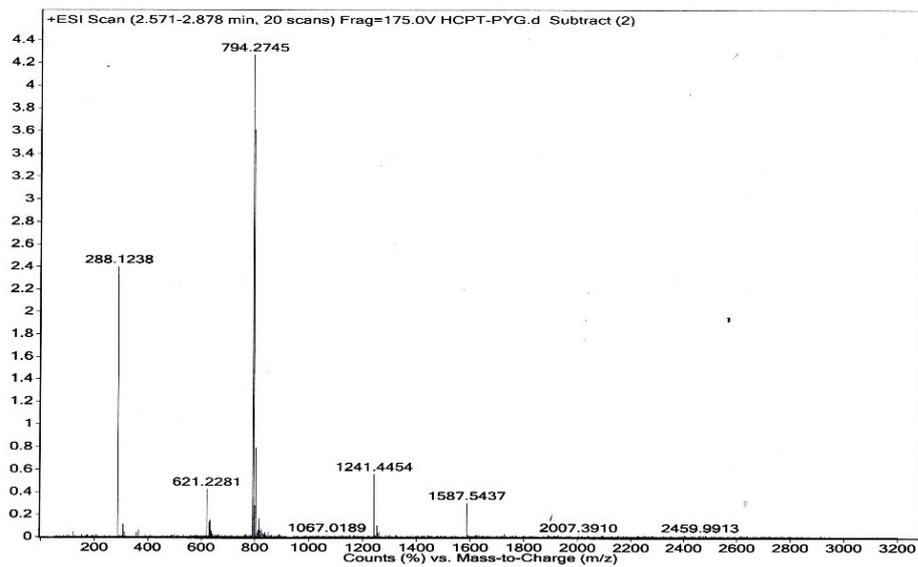


**Figure S4.** HR-MS spectrum of **Comp. 2**.

## Compound 4:

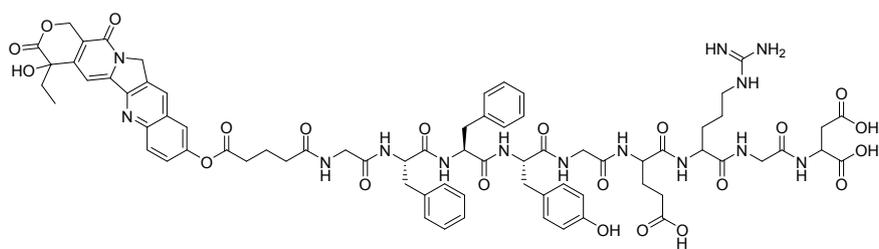


HR-MS: calc  $M^+ = 1585.5391$ , obsvd  $(M+2H)^{2+} = 794.2745$ .

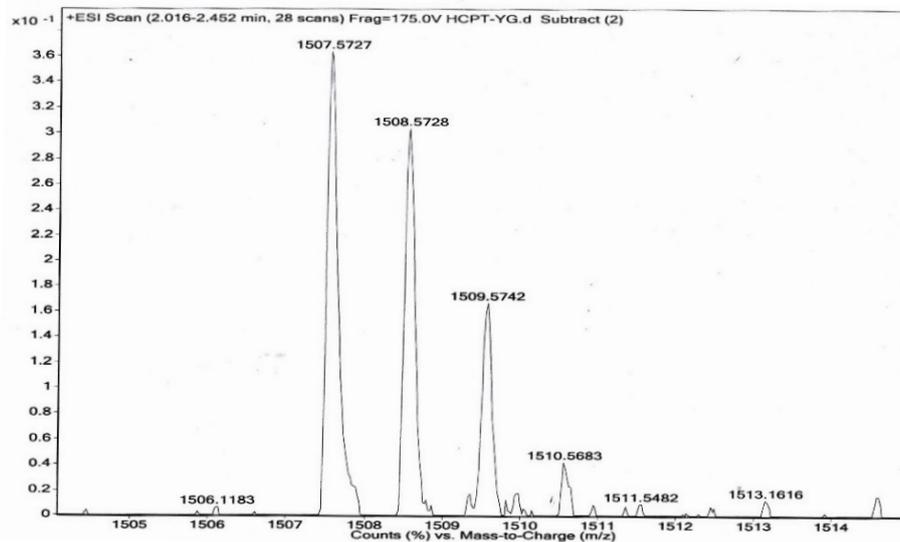


**Figure S5.** HR-MS spectrum of *Comp. 4*.

**Compound 5:**

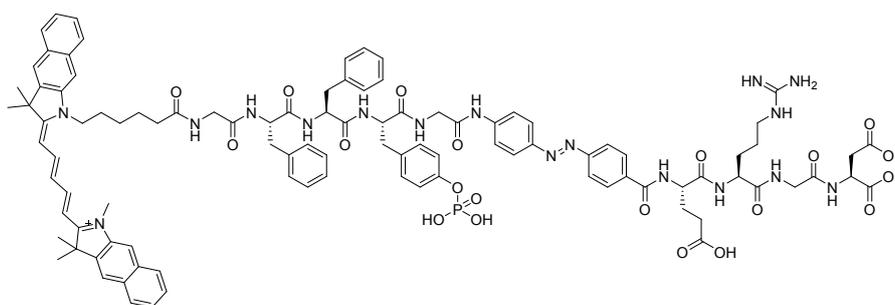


HR-MS: calc  $M^+ = 1506.5728$ , obsvd  $(M+H)^+ = 1507.5727$ .

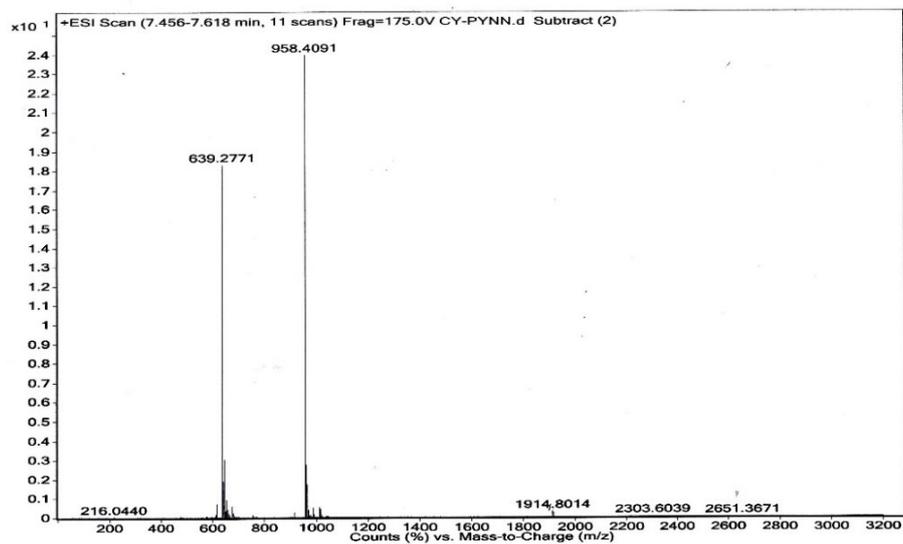


**Figure S6.** HR-MS spectrum of *Comp. 5*.

**Compound 6:**

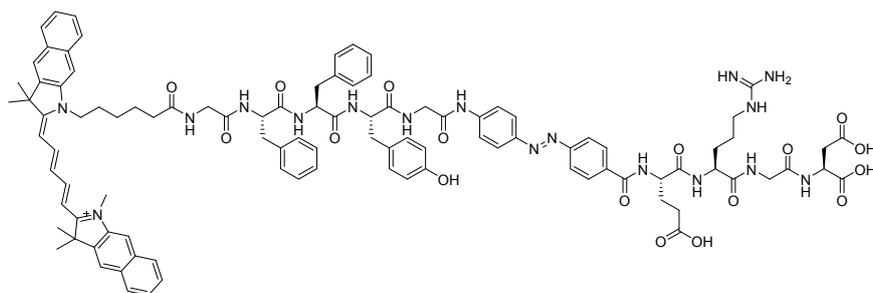


HR-MS: calc  $M^+ = 1915.8113$ , obsvd  $(M+2H)^{2+} = 958.4091$ .

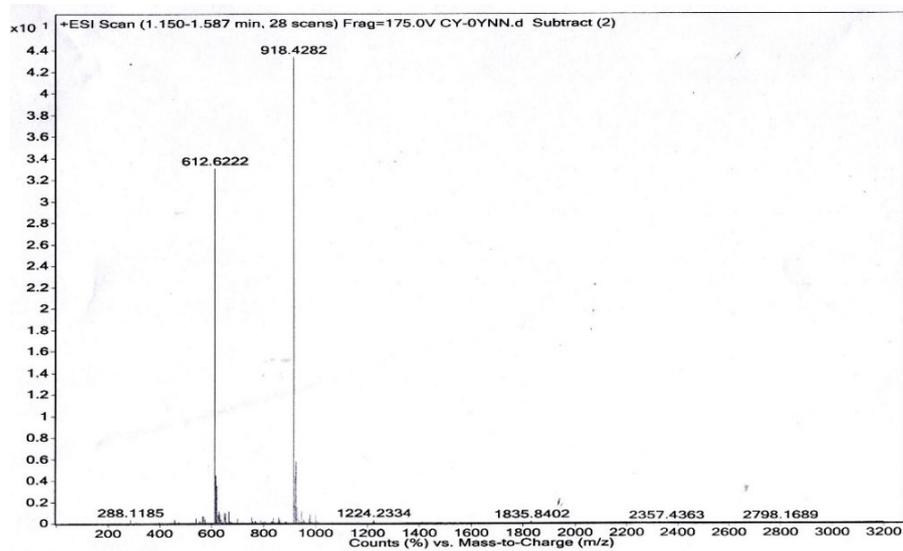


**Figure S7.** HR-MS spectrum of *Comp. 6*.

**Compound 7:**

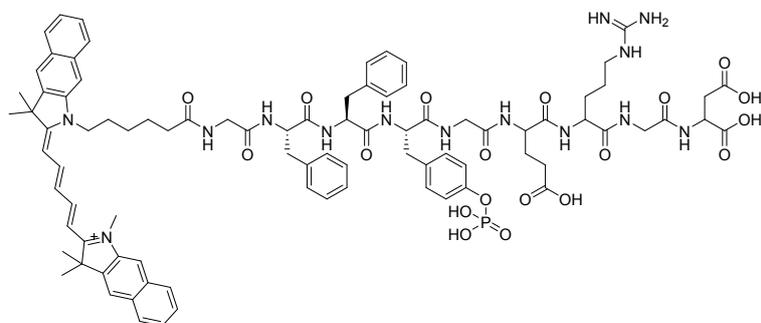


HR-MS: calc  $M^+ = 1835.8450$ , obsvd  $(M+2H)^{2+} = 918.4282$ .

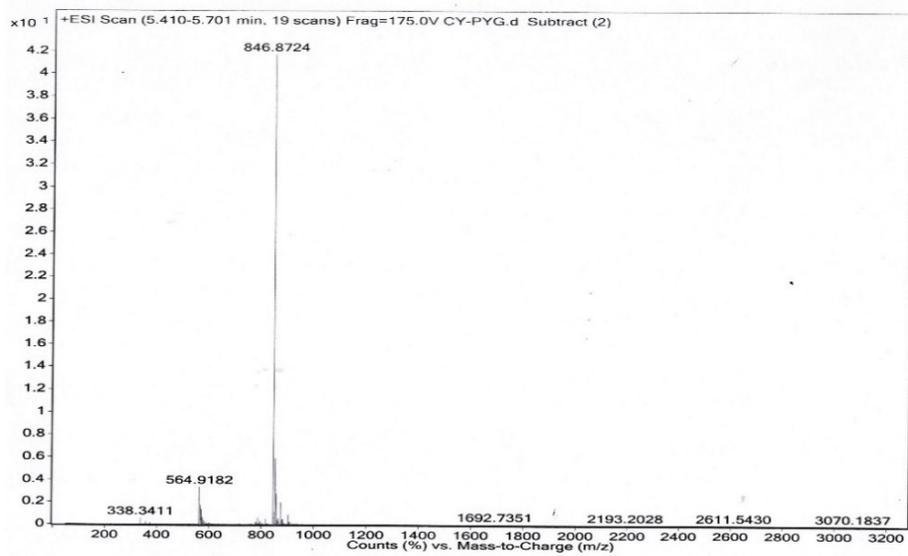


**Figure S8.** HR-MS spectrum of *Comp. 7*.

**Compound 8:**

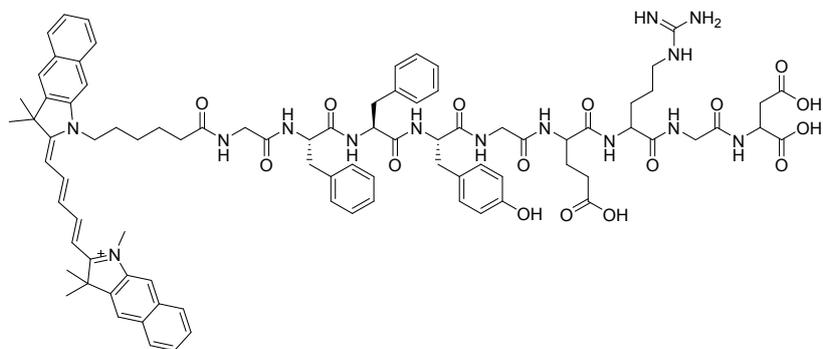


HR-MS: calc  $M^+ = 1691.7334$ , obsvd  $(M+2H)^{2+} = 846.8724$ .

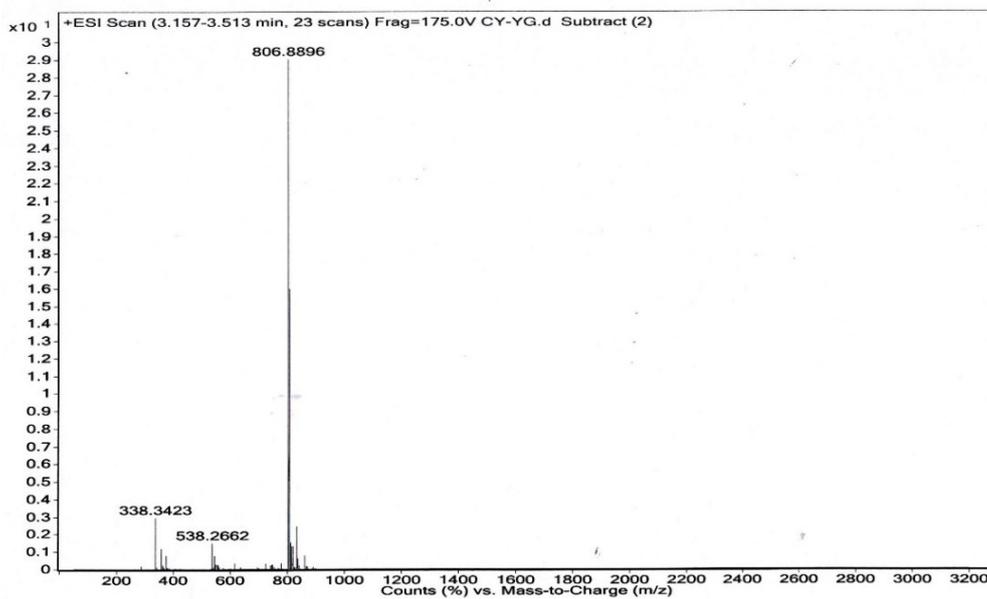


**Figure S9.** HR-MS spectrum of **Comp. 8**.

**Compound 9:**



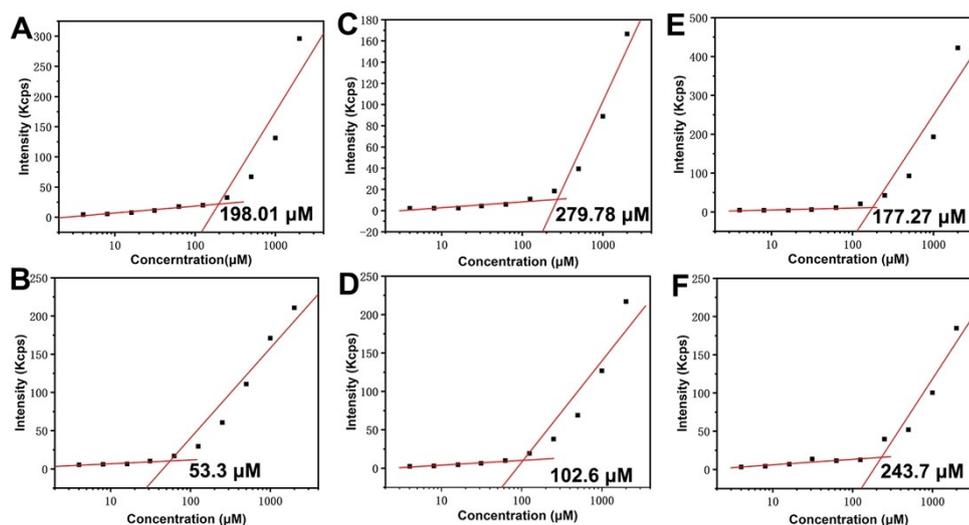
HR-MS: calc  $M^+$  = 1611.7671, obsvd  $(M+2H)^{2+}$  = 806.8896.



**Figure S10.** HR-MS spectrum of *Comp. 9*.

### **Critical aggregation concentration**

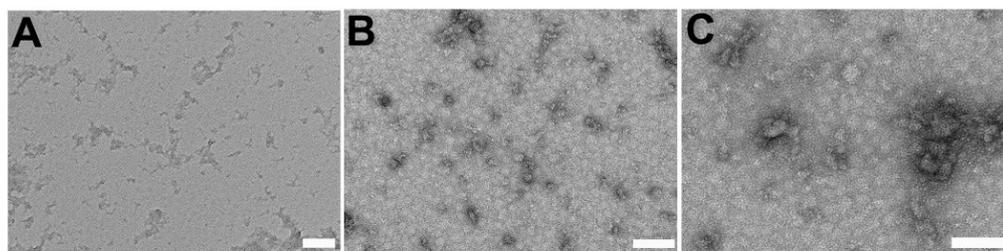
Compound 1 and compound 4 incubated with ALP (10 U/mL) until completely converted detected by LC-MS, respectively. The CAC value of compound 1, 2, 4, 5, compound 1 with ALP and compound 4 with ALP in PBS solution (pH = 7.4) were determined by dynamic light scattering (DLS), and the light scattering intensity was recorded for each concentration analyzed.



**Figure S11.** Critical aggregation concentration of A) *Comp. 1*, B) *Comp. 1* + ALP (10 U/mL), C) *Comp. 4*, D) *Comp. 4* + ALP (10 U/mL), E) *Comp. 2*, F) *Comp. 5*.

### Transmission electron microscopy

10 μL of samples were added to the carbon-coated copper grids, excess samples were removed with filter paper, then uranyl acetate for negative staining. At last, sample were placed in the desiccator overnight and the observed with transmission electron microscopy.

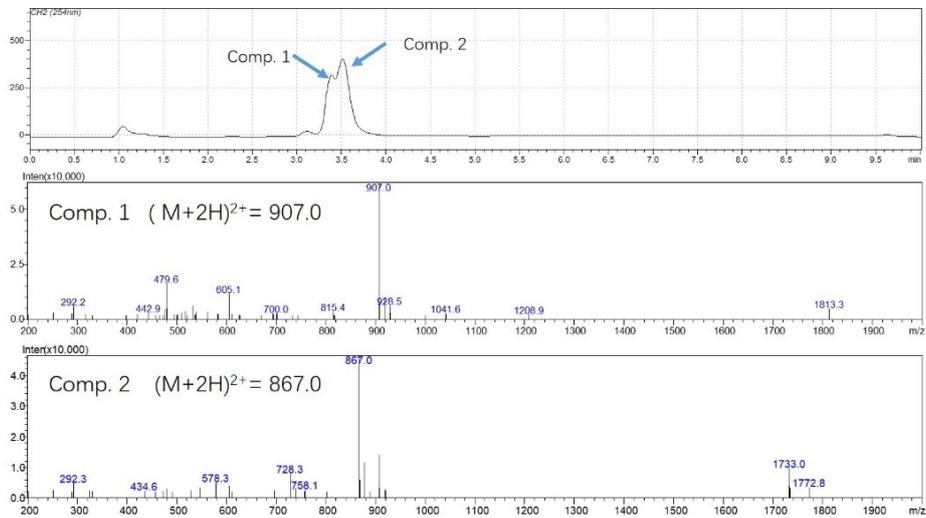


**Figure S12.** TEM images of A) *Comp. 1* in PBS, B) and C) *Comp. 4* + ALP (10 U/mL), scale bar = 100 nm.

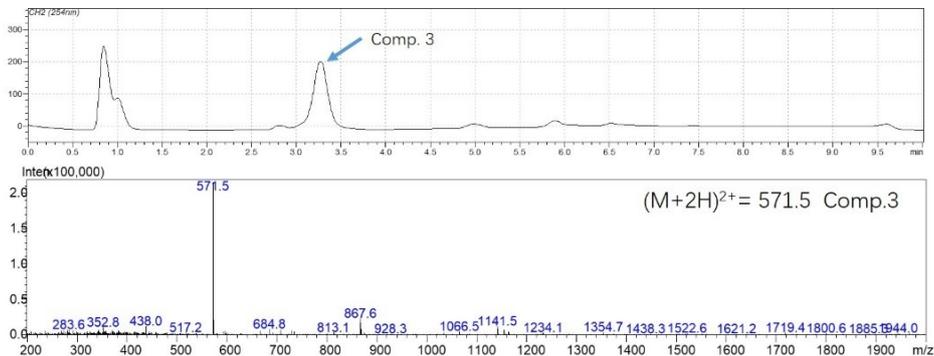
### Tandem molecular self-Assembly of *Comp. 1*

1.8 mg of *Comp. 1* was dissolved in 994 μL of PBS (pH=7.4) as a stock solution. The concentration of the work solution was 100 μM. Na<sub>2</sub>CO<sub>3</sub> (1 M) was added to the above solution to adjust the final pH to 7.4. The solution was incubated at 37 °C. The Alkali phosphatase (ALP, 10 U/mL) was then added to the solution for 10 hours at 37

°C to trigger the first step of self-assembly. After that, Sodium Dithionite (SDT, 20mM) were added for 2 hours at 37 °C to trigger the second step of self-assembly.



**Figure S13.** LC traces to show the conversion from *Comp. 1* to *Comp. 2* by adding ALP for 3 h and mass spectrum of *Comp. 1 and Comp. 2* detected in above process.



**Figure S14.** LC traces to show the conversion from *Comp. 2* to *Comp. 3* by adding SDT (20 mM) for 2 h and mass spectrum of *Comp. 3* detected in above process.

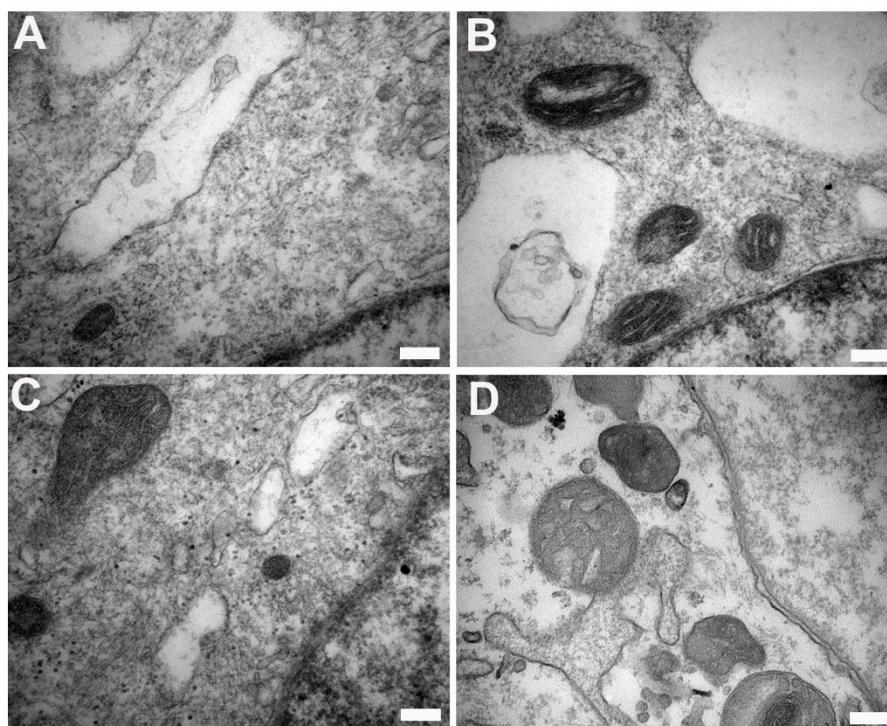
## Cell experiments

LO2, PC-3, MCF-7, HeLa, and A549 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), LO2, PC-3, MCF-7, HeLa and A549 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), 100 U/mL penicillin

and 100 µg/mL streptomycin. All cells were at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Bio-TEM

A549 cells was seeded in 6 well cultural dish at a density of  $2 \times 10^5$  cells. After incubation for 18 h, medium was removed. The cells were then incubated with *Comp. 1* or *Comp. 2, 4, 5* (100 µM) for 6 h. The culture medium was removed, the cells were collected using a cell scraper and then fixed with glutaraldehyde (2.5%), and finally observed by transmission electron microscope (TEM).



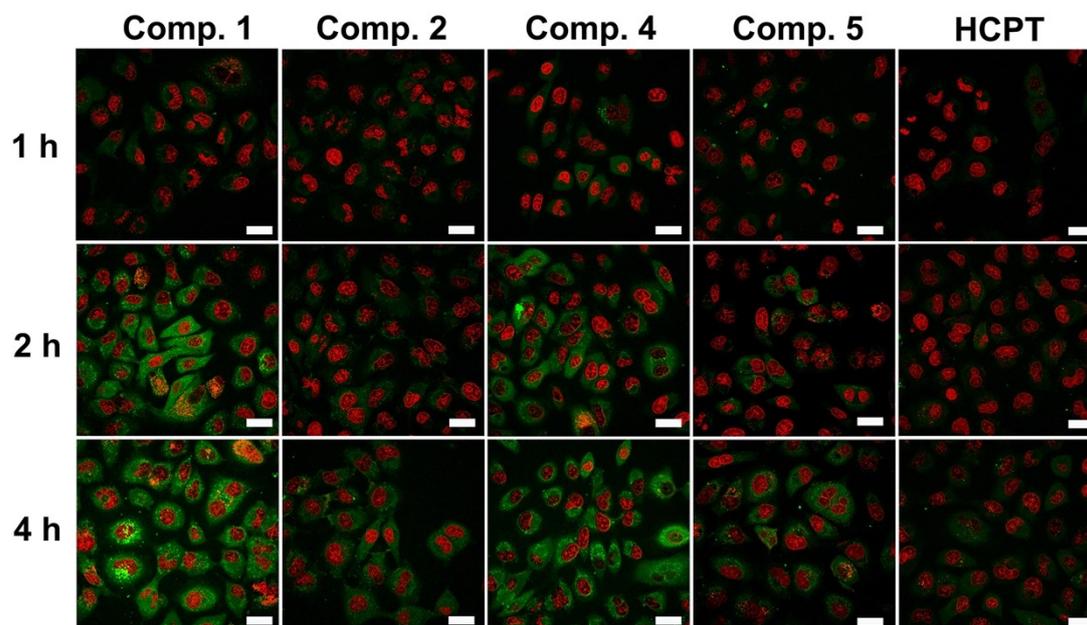
**Figure S15.** Bio-TEM image of A549 cells treated by A) *Comp. 2*, B) *Comp. 4*, C) *Comp. 5* and D) blank, white scale bar = 200 nm.

### Confocal Laser Scanning Microscope experiments

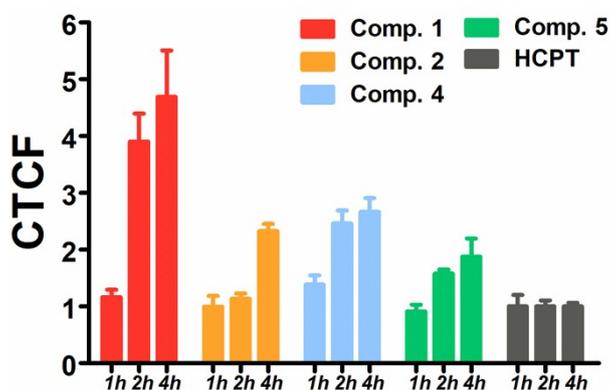
#### Cell uptake

A549 cells was seeded in CLSM cultural dish at a density of  $1 \times 10^5$  cells. After incubation for 18 h, medium was removed. The cells were then incubated with *Comp. 1, 2, 4 or 5* (100 µM) for 1, 2, 4 and 6 h, respectively. The medium was removed and the cells incubated with Red dot 1 (1X, diluted with DMEM) for 5-10 min. Wash cells with

DMEM for three times, we then used CLSM to observe fluorescence intensity and used Image J to analyze relative fluorescence intensity. ( $\lambda_{ex}=405\text{ nm}$ , all test conditions are consistent.)



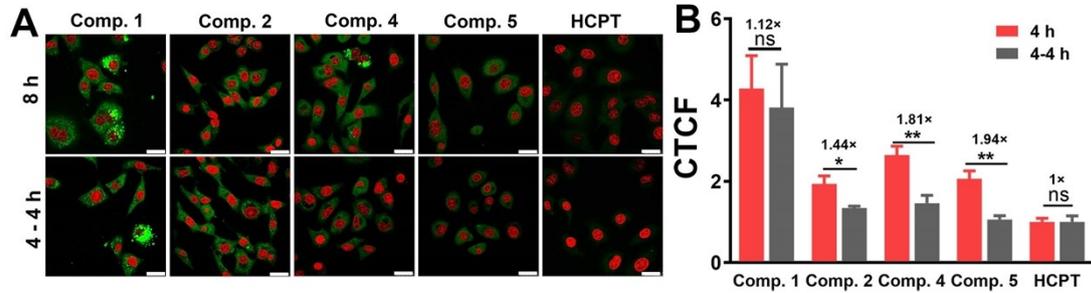
**Figure S16.** CLSM images of A549 cells incubated with *Comp. 1, 2, 4, 5* and HCPT (100  $\mu\text{M}$ ) for 1, 2 and 4 h.



**Figure S17.** Corrected total cell fluorescence (CTCF, quantified from the gray scale of **Figure S15**).

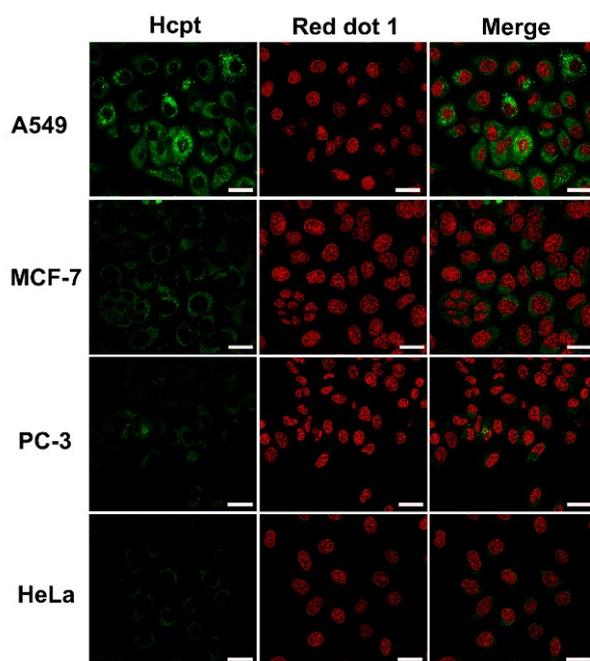
A549 cells was seeded in CLSM cultural dish at a density of  $1 \times 10^5$  cells. After adhesion, medium was removed. For 4-4 h group, the cells were then incubated with *Comp. 1, 2, 4* or *5* (100  $\mu\text{M}$ ) for 4 h. Then medium containing compound was removed and added fresh medium the cells further incubation for 4 h. The medium was removed and the cells incubated with Red dot 1(1X, diluted with DMEM) for 5-10 min. As comparson (4 h), the cells were then incubated with *Comp. 1, 2, 4* or *5* (100  $\mu\text{M}$ ) for 4

h. The medium was removed and the cells incubated with Red dot 1(1X, diluted with DMEM) for 5-10 min. Wash cells with DMEM for three times, we then used CLSM to observe fluorescence intensity and used Image J to analyze relative fluorescence intensity. ( $\lambda_{ex}= 405 \text{ nm}$ , all test conditions are consistent.)

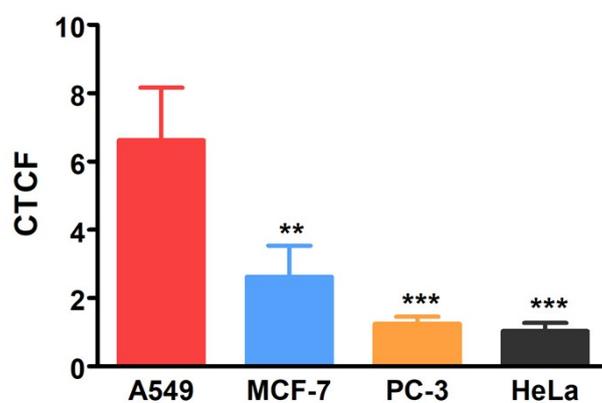


**Figure S18** A) CLSM images of A549 cells treated by different means. B) Corrected total cell fluorescence (CTCF, quantified from the gray scale of A, analyzed by t test,  $***P < 0.001$ ,  $**P < 0.01$ ,  $*P < 0.05$ ,  $N = 5$ ), the relative ratio number obtained by the ratio of means.

Cells, including LO2, PC-3, MCF-7, HeLa, and A549, were seeded in CLSM cell culture plate at a concentration of  $1 \times 10^5$  cell. After incubation for 24 h, upper medium removed then cell incubated with **Comp.1** (100  $\mu\text{M}$ ) for 6 h. The medium was removed and the cells incubated with Red dot 1(1X, diluted with DMEM) for 5 mins to stain cell nuclues. Wash cells with DMEM for three times, we then used CLSM to observe fluorescence intensity and used Image J to analyze relative fluorescence intensity. ( $\lambda_{ex}= 405 \text{ nm}$  , all test conditions are consistent.)



**Figure S19.** CLSM images of *Comp. 1* (100  $\mu$ M) incubated with A549, MCF-7, PC-3 and HeLa for 6 h (HCPT:  $\lambda_{exc}$  = 405 nm, emission = 470 - 490 nm. Reddot1:  $\lambda_{exc}$  = 633 nm, emission = 650 - 750 nm, white scale bar = 25  $\mu$ m).

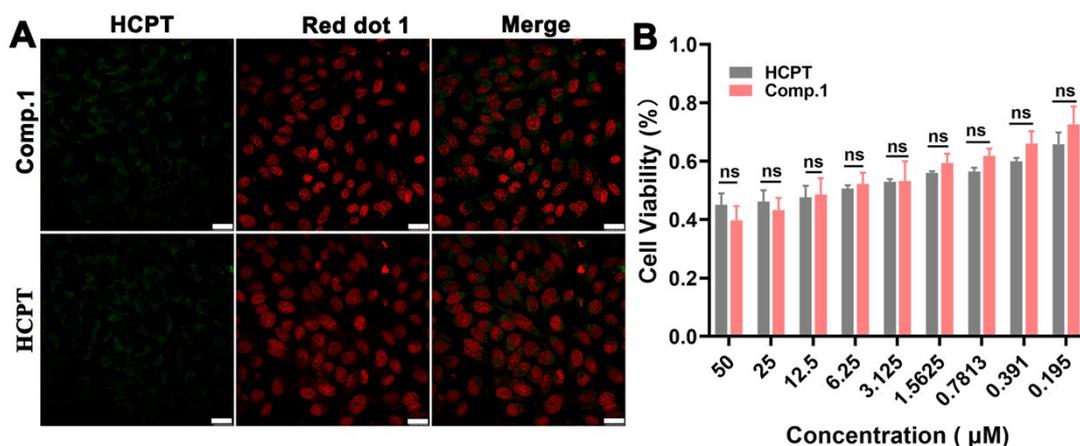


**Figure S20.** Corrected total cell fluorescence (CTCF, quantified from the gray scale of **Figure S19**, One-way ANOVA, compared to A549 group, Mean $\pm$ SD, \*\*\*P < 0.001, \*\*P < 0.01. N = 5).

### Cell proliferation

All of cell lines were incubated in 96-well plate at a density of about 6000. Incubate with compound for 48 hours after cell attachment. Remove the medium containing compound and cell was fixed by 100  $\mu$ L cold trichloroacetic acid solution (10%) at 4  $^{\circ}$ C overnight. Then discarding the fixative solution and washed by water for

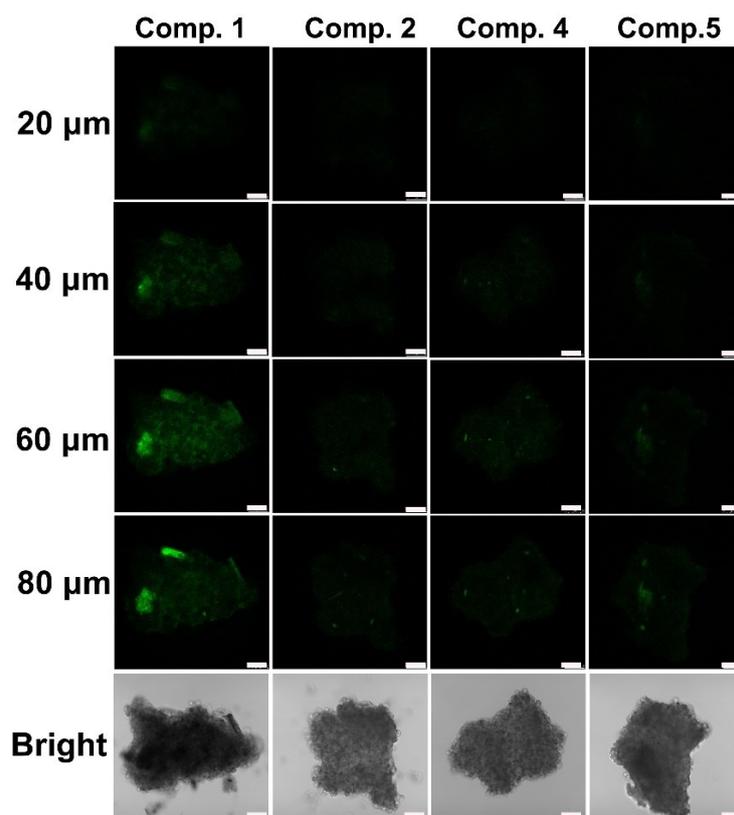
three times, put the 96-well plate in a 37 ° C oven to dry. Next, 0.4%SRB solution (100µL) was used to stain cell for 25 min at room temperature to form SRB-bound protein, then using 300 µL AcOH solution (1% ) to clean excessive SRB. Put the 96-well plate in a 37 ° C oven to dry and add 10 mM Tris base solution of 100 µL every well to solubilize the SRB-bound protein for 30 min. Measure the OD at 570 nm in microplate reader.



**Figure S21.** A) CLSM images of Comp. 1 and HCPT (100 µM) incubated with LO2 cells for 6 h (HCPT:  $\lambda_{exc}$  = 405 nm, emission = 470-490 nm. Reddot1:  $\lambda_{exc}$  = 633 nm, emission = 650-750 nm, white scale bar = 25 µm). B) cells viability values for **Comp. 1** and HCPT towards LO2 cells. The data is presented as the mean  $\pm$  SEM and analyzed by t test, \*\*\*P<0.001, \*\*P<0.01, \*P<0.05, n=3.

### Multicellular Cell Spheroids.

To prepare the three-dimensional cell spheroids model, heated 100 µL of sterile 1.5 % agarose (w/v) solution was dispensed into 96-well plates and cooled at room temperature for solidification. The suspension of A549 cells was seeded on the gel at a concentration of  $3 \times 10^3$  cells per well and incubated at 37 °C for 6 days to form the multicellular Cell spheroids (MCSs). To evaluate the tissue penetration ability in vitro, the A549 MCSs were treated with **Comp. 1, 2, 4** and **5** at 37 °C for 6 h at concentration of 100 µM. After that, the tumor spheroids were washed with PBS for 3 times and observed under CLSM. ( $\lambda_{exc}$ = 405 nm, all test conditions are consistent.)



**Figure S22.** CLSM microscopy images of A549 MCSs incubated with *Comp. 1*, *Comp. 2*, *Comp. 4*, and *Comp. 5* (100  $\mu\text{M}$ ) for 6 h, scale bar = 100  $\mu\text{m}$ .

### The subcutaneous tumor model.

BALB/c nude mice (male,  $20 \pm 2$  g, 6-8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). All animal experiments were carried out according to the guidelines of animal ethics committee of Nankai University. A549 tumor-bearing nude mice model was established by subcutaneously inoculating with A549 cells ( $4 \times 10^6$  per mouse) mixed with growth factor Matrigel (50  $\mu\text{L}$ , BD Biosciences) at the right flank. After 10 days, tumor volumes reached about 100  $\text{mm}^3$  for subsequent tests.

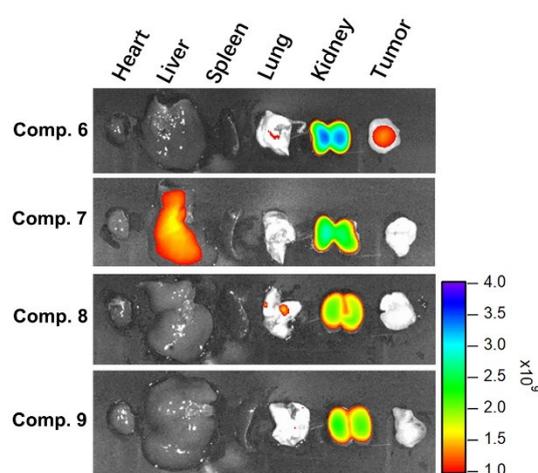
### Living animal image

To investigate the accumulated efficiency *in vivo*. Tumor-bearing mice were randomly divided into 4 groups. Cy5.5-labeled peptide compounds were injected into mice through the tail vein when tumor volumes reached about 200  $\text{mm}^3$ . Anesthetize

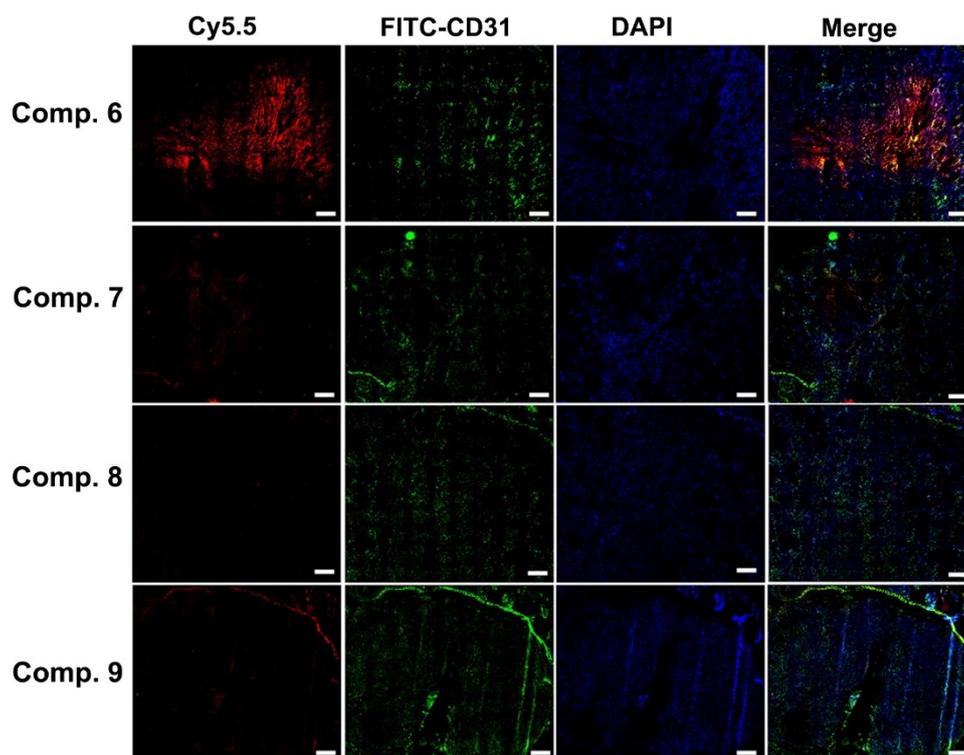
the mice with isoflurane at the specified time point for intravital imaging via IVIS living image system. The injection dosage of the compound is shown in the table below.

Group	Compound	Dose
1	<i>Comp. 6</i>	16.5 mg/kg, 200 $\mu$ L/mouse
2	<i>Comp. 7</i>	15.75 mg/kg, 200 $\mu$ L/mouse
3	<i>Comp. 8</i>	14.5 mg/kg, 200 $\mu$ L/mouse
4	<i>Comp. 9</i>	13.75 mg/kg, 200 $\mu$ L/mouse

**Table S1.** Compound formulations and doses for evaluation of accumulated efficiency.



**Figure S23.** Representative ex vivo fluorescence images of excised organs and tumor at 36 h



**Figure S24.** CLSM images of cryo-sections of A549 tumor tissue after intravenous administration of *Comp. 6*, *Comp. 7*, *Comp. 8* and *Comp. 9* for 24 h, including DAPI-stained nucleus (blue channel), FITC-CD31-stained blood vessel (green channel), Cy5.5 (red channel), and overlay image, scale bar = 250  $\mu\text{m}$ .

#### **Tumor penetration study *in vivo*.**

BALB/c nude mice bearing A549 tumor were intravenously injected with *Comp. 6*, *Comp. 7*, *Comp. 8* and *Comp. 9* (dose displayed in table S1). After 24 h, mice were sacrificed and tumor was separated for frozen sections. The tumor samples were sectioned into 8  $\mu\text{m}$  thickness at equatorial plane. Then samples were fixed in acetone for 15 min, washed twice with PBS, 5% BSA blocking solution prevented nonspecific binding at room temperature for 1 h and incubated with FITC-CD31 antibody for 1 h. After washed with PBS for 3 times, samples were stained with DAPI for 10 min.

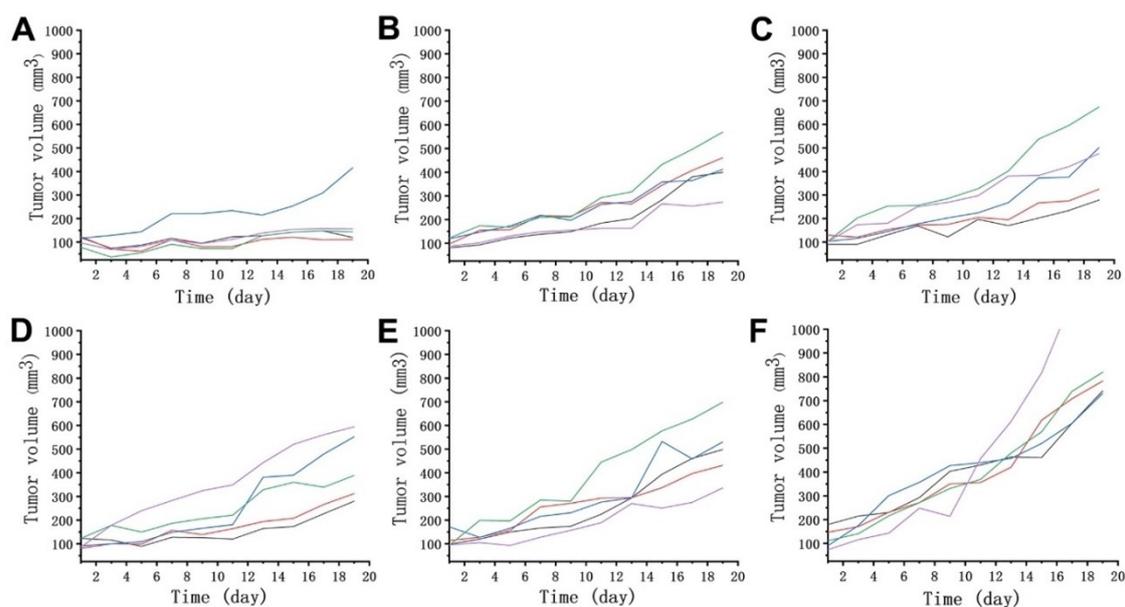
#### **Anti-tumor therapy.**

The mice were randomly into six groups ( $n = 5$ ). A549 tumor-bearing nude mice were intravenously injected with *Comp. 1*, *Comp. 2*, *Comp. 4*, *Comp. 5*, free HCPT and

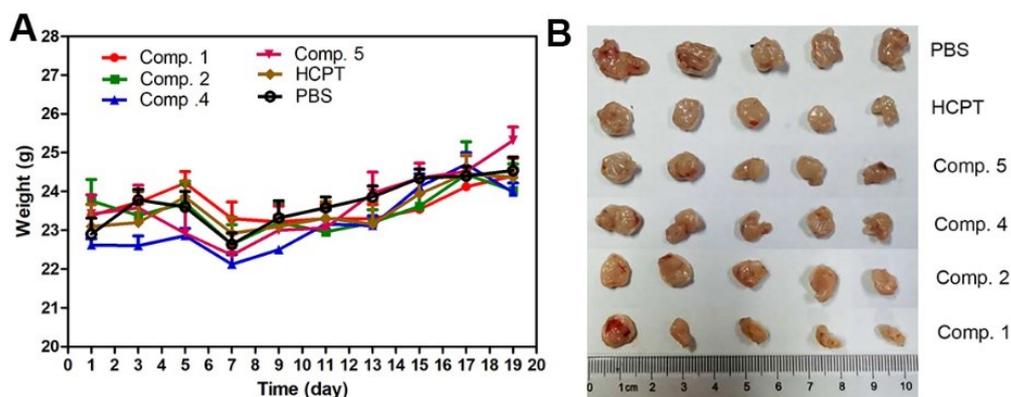
PBS at 1, 4, 7 and 10 day. Tumor volume and weight were monitored per 2 days for 19 days. After the whole observation period, mice were sacrificed, and solid tumor was separated, fixed in 4% formaldehyde for hematoxylin and eosin (H&E) staining and immunofluorescence analysis.

Group	Compound	Dose
1	<i>Comp. 1</i>	14.90 mg/kg, 200 $\mu$ L/mouse
2	<i>Comp. 2</i>	14.26 mg/kg, 200 $\mu$ L/mouse
3	<i>Comp. 4</i>	13.07 mg/kg, 200 $\mu$ L/mouse
4	<i>Comp. 5</i>	12.41 mg/kg, 200 $\mu$ L/mouse
5	HCPT	3mg/kg, 200 $\mu$ L/mouse
6	PBS	200 $\mu$ L/mouse

**Table S2.** Therapy agents and doses for evaluation of anti-tumor.



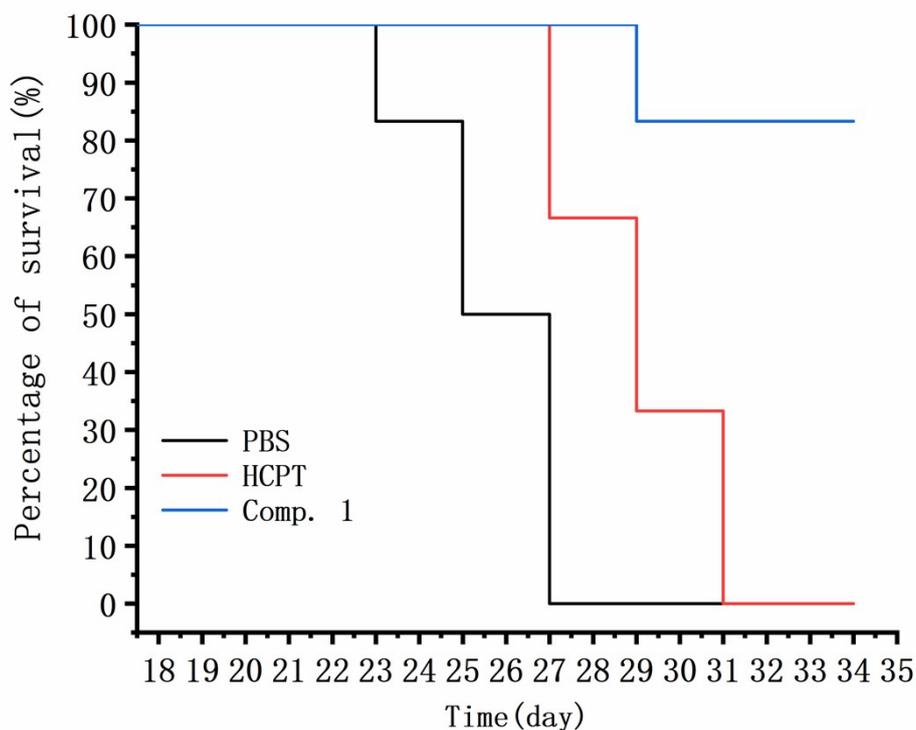
**Figure S25.** Single component tumor volume curves of A) *Comp. 1*, B) *Comp. 2*, C) *Comp. 4*, D) *Comp. 5*, E) HCPT, F) PBS



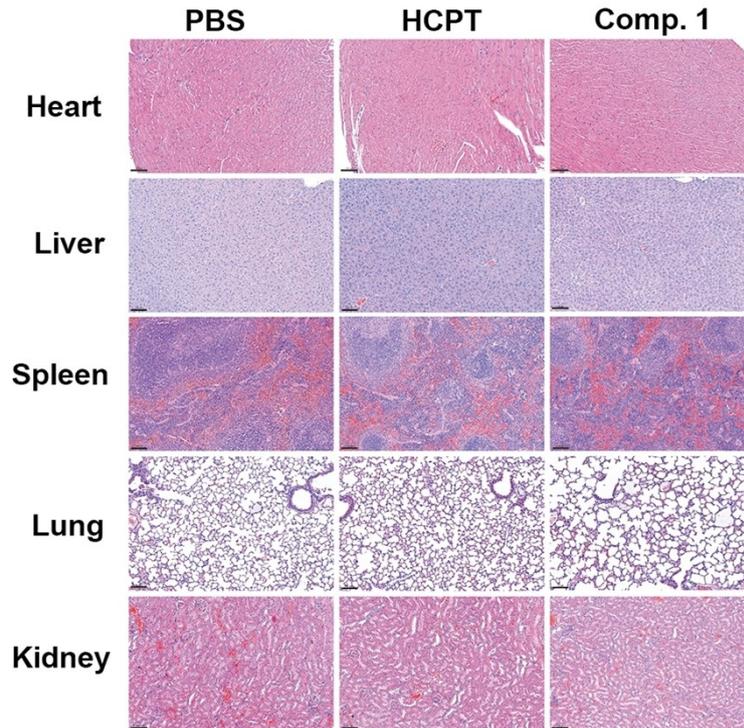
**Figure S26.** A) Body weight curve of treatment mouse, B) optical image of tumor

### Survival rate evaluation

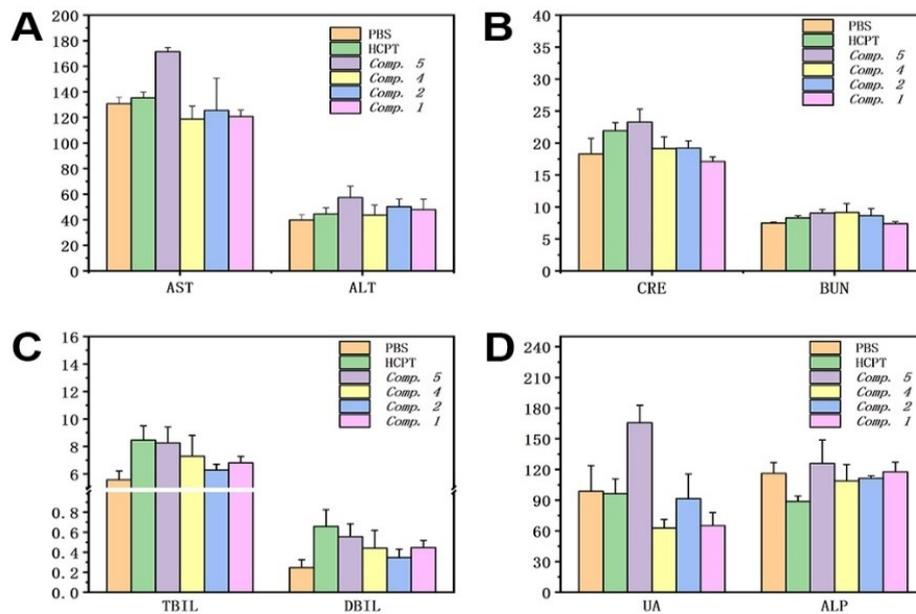
The mice were randomly into three groups (n = 6). A549 tumor-bearing nude mice were intravenously injected with **Comp. 1**, free HCPT and PBS at 1, 4, 7 and 10 day. Tumor volume were monitored per 2 days until the tumor volume exceeds 1000 mm<sup>3</sup>.



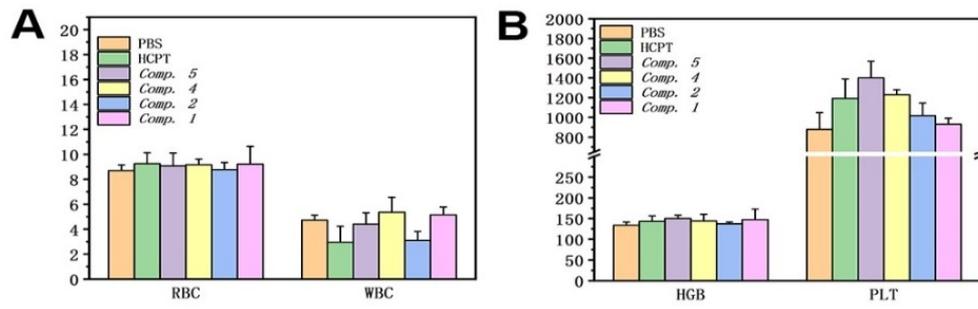
**Figure S27.** Survival curves of the A549 tumor-bearing nude mice (n = 6)



**Figure S28.** Representative images of H&E-stained heart, lung, liver, spleen and kidney slices excised from mice after completing treatment, black bar = 100  $\mu$  m.



**Figure S29.** A) aspartate transaminase (AST, U/L); alanine transaminase (ALT, U/L) . B) creatinine (CRE, umol/l); blood urea nitrogen (BUN, mmol/l). C) total bilirubin (TBIL, umol/l); direct Bilirubin (DBIL, umol/l). D) urea (UA, umol/l); alkaline phosphatase (ALP, U/L). n = 3.



**Figure S30.** A) red blood cell (RBC,  $10^9/L$ ); white blood cell (WBC,  $10^9/L$ ) . B) hemoglobin (HGB, g/L); platelets (PLT,  $10^9/L$ ), n = 4.

**Reference:**

1. D. Zheng, Y. Chen, S. Ai, R. Zhang, Z. Gao, C. Liang, L. Cao, Y. Chen, Z. Hong, Y. Shi, L. Wang, X. Li and Z. Yang, *Research (Wash D C)*, 2019, **2019**, 4803624.