Supporting Information

A dual-targeted trinity of antibody-peptide-drug delivery consortium to combat HER2+ tumor

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1. Materials and methods

1.1 Materials

Rink Amide Resin (1.1 mmol/g) was obtained from Nankai University resin Co., Ltd. Fmoc-amino acids and o-benzotriazol-1-yl-N,N,N',N'-tetramehtyluronium hexafluorophosphate (HBTU) were bought from GL Biochem (Shanghai). 10-Hydroxy camptothecin (HCPT, purity ≥98%) were purchased from Baoman Biological technology co. (Shanghai, China). Chemical regents and solvents were used as received from commercial sources. High-glucose DMEM Basal Medium, Fetal Bovine Serum, 0.25% Trypsin-ethylene diamine tetraacetic acid (EDTA) solution, and Penicillin-Streptomycin Solution were purchased from Gibco (USA). CCK-8 Assay Kit was bought from MCE (Shanghai, China). SKBR-3, NCl-N87, HCC-70 and PC-3 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Eight-week-old male BALB/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

1.2 General methods

The ^{1}H NMR and HR-MS of the compounds were performed by Bruker ARX-400 and Agilent 6520 Q-TOF LC/MS with DMSO-d_6 as solvent. The peptide derivatives were purified by High Performance Liquid Chromatography (HPLC, Germany) system using the C18 reversed phase column with methanol containing 0.05% TFA and water containing 0.05% TFA as eluents. The morphology of the peptide derivatives was observed by transmission electron microscopy (TEM) on the Tecnai G2 F20 system at an operating voltage of 200 kV. Critical aggregation concentration (CAC) values were determined by dynamic light scattering (DLS), these experiments conducted on a laser light scattering spectrometer (BI-200SM, Brookhaven, USA). Reology (TA instrument) test was done on an AR 2000ex system, the 25 mm parallel plate was used during the experiment at the gap of 500 μm. Circular dichroism (CD) spectrum was obtained by a BioLogic (MOS-450) system. The fitting curve of microscale thermophoresis (MST) was performed using the Monolith NT.115 system (NanoTemper Technologies, Germany). The K_D value was calculated using the NanoTemper software package. Cellular uptake and drug tracking images were taken by a confocal laser scanning microscopy (Leica TSC SP8, Germany).

1.3 Preparation of peptide derivatives

All peptide derivatives were prepared using standard peptide solid phase synthesis (SPPS) method. Fmoc-Lys(Mtt)-OH, Fmoc-Glu-OtBu, Boc-Glu-OtBu, and the corresponding N-Fmoc-protected other amino acids were added to the system. Benzotriazole-N, N,N′,N′ - tetramethyluronium hexafluorophosphate (HBTU) was used as the coupling reagent to couple with the free amino group of the next Fmoc-protected amino acid. The Rink amino resin first was used to synthesize Hcpt-GFFYKRGD. The side chain of lysine was protected with 4-methyl trityl (Mtt), 1% trifluoroacetic acid (TFA) containing 2.5% triisopropylsilane (TIS) and 2.5% H_2O can

take off Mtt group without removing peptide from the resin. Then Fmoc-Glu-OtBu and Boc-Glu-OtBu were conjugated to lysine side chain amino. The peptide Hcpt-GFFYKRGD(γE)$_2$-NH_2 finally was cut off from the resin with 95% TFA. Next, ice-cold anhydrous diethylether was added to the lysis reagent, the solvent was evaporated, and the resulting precipitate was the crude peptide derivative. The crude peptide derivative was separated and purified by reversed-phase high performance liquid chromatography (HPLC), and the structure of the compound was characterized by high resolution mass spectrometry (HR-MS) and ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR).

1.4 Transmission electron microscopy (TEM)

10 μL of ***Comp. 1***, ***Comp. 2***, ***Comp. 1***+Herceptin, and ***Comp. 2***+Herceptin at the concentration of 1 mM was added to the carbon-coated copper grid, and the samples were washed twice with ultrapure water. After further removing excess water with filter paper, the samples were negatively stained with uranyl acetate. The copper grid was placed in a desiccator until it was completely dry. The samples were observed by transmission electron microscope (TEM).

1.5 Critical aggregation concentration (CAC)

The CAC values of ***Comp. 1***, ***Comp. 2***, ***Comp. 1***+Herceptin, and ***Comp. 2***+Herceptin were determined by dynamic light scattering (DLS) at 37 ℃. Solutions containing different concentration of ***Comp. 1***, ***Comp. 2***, ***Comp. 1***+Herceptin, and ***Comp. 2***+Herceptin were tested and the light scattering intensity corresponding to each concentration was recorded. Each set of experiments was repeated three times and averaged.

1.6 Rheology

We dispersed ***Comp. 1*** or ***Comp. 2*** in PBS at a concentration of 0.5 wt%, and then added 10 wt% of Herceptin to the solution. The resulting solutions were immediately added between 25 mm parallel plates for rheology test. The mode of time sweep was tested at the strain of 1% and frequency of 1% at 37℃. The samples were also characterized by the mode of dynamic time sweep in the region from 0 min to 60 min.

1.7 Circular dichroism (CD) spectrum

The ***Comp. 1***, ***Comp. 2***, ***Comp. 1***+Herceptin, and ***Comp. 2***+Herceptin were added to the 0.1 cm quartz spectrophotometer cell (20-C/Q/0.1) for CD spectrum test. The wavelength was set from 185 to 280 nm, and the acquisition period was 0.5 s and the step length was 0.5 nm. A BioLogic (mos-450) system was used to record the CD spectra. The final spectrum was subtracted from the PBS background.

1.8 Microscale thermophoresis (MST)

Herceptin was labeled with the fluorescent dye NT-647 using Monolith NT™ Protein Labelling Kits (cysteine-reactive) (NanoTemper Technologies, Germany). PBS buffer

containing 0.05% Tween-20 (pH = 7.4) was used as the assay buffer. For the interaction experiments of fluorescent-proteins with ***Comp. 1*** or ***2***, the concentration of labelled Herceptin was maintained constant, while the concentration of ***Comp. 1*** or ***2*** varied from 0.14 μM to 4.5 mM. Then the solution of fluorescent-proteins was mixed with solutions containing different concentrations of ***Comp. 1*** or ***2*** at 1:1 volume ratio. After a short incubation time, the samples were loaded into MST NT.115 standard glass capillaries and the analysis was performed using the Monolith NT.115 system (NanoTemper Technologies, Germany). The K_D value was calculated using the NanoTemper software package.

1.9 Cell culture

Cells were planted in Dulbecco's Modified Eagle Medium (DMEM) which was enriched with 10% fetal bovine serum (FBS) and 1% (volume/volume) Penicillin-Streptomycin Solution. The cells were then cultured at a temperature of 37 °C in a humidified atmosphere consisting of 95 % air and 5 % CO_2. Upon reaching a confluence of 80% to 90%, the cells were detached using a 0.25 % trypsin-ethylene diamine tetraacetic acid solution. The growth medium was replaced every three days.

1.10 Preparation of FITC-labeled Herceptin

1 mg/mL of Herceptin and 1 equiv. of fluorescein isothiocyanate (FITC) were mixed in a dialysis bag and placed at 4 °C for stirring overnight. The label solution in the dialysis bag was removed, immediately filtered with SephadexG50 gel, the free fluorescein was removed, subpackaged, and stored at 4 °C.

1.11 AutoDock

The docking simulation is performed by implementing the AutoDock Vina Software (version 1.1.2) for the ***Comp. 1***/Herceptin complex[1].

1.12 Confocal laser scanning microscopy (CLSM) image

Cells in exponential growth phase were seeded in cell culture chamber at 1×10^5 cell/well. The cells were allowed for attachment for 12 h at 37 °C, 5% CO_2. The culture medium was removed, and new culture medium containing Comp. ***1***+Herceptin, or Comp. ***2***+Herceptin was added, respectively. Under dark conditions, use Red Dot 1 for nuclear staining. After incubation for 1 h or 4 h, cells were rinsed 5 times by PBS buffer, and then kept in the live cell imaging solution (gibco ® by Life TechnologiesTM, A14291DJ) for imaging by a Live cell Imaging System.

1.13 Cell viability assay

Cells (8×10^3/well) were seeded in 96-well plates and incubated with growth medium for 12 h. The viability of cells harvested on 48 h after treatment with different drugs was assessed using CCK-8 Kit (MCE; cat. HY-K0301). At designated time point, the growth medium was discarded and replaced with serum-free DMEM medium supplement with 10% CCK-8 and cultured for 2 h, and then the absorbance at the 450 nm wavelength was measured using microplate reader. The cytotoxicity assay was

performed 3 times and the average value of the three measurements was taken. To ensure the reproducibility for determining of the IC_{50} of different drugs, we collected the cytotoxicity of different drugs with at least 10 concentration gradients for three separate experiments and average all the experiments, finally, the IC_{50} values of different drugs were calculated by SPSS.

1.14 Synergistic Effect Evaluated by Combination Index (CI)

The CI of ***Comp. 2*** and Herceptin when combined in ***Comp. 2***+Herceptin was calculated from the IC_{50} values according to the equation: CI = D/Dm1 + D/Dm2, where D is the IC_{50} value of ***Comp. 2***+Herceptin, that in combination produce a certain level of cytotoxicity, and Dm1 and Dm2 are the IC_{50} values of ***Comp. 2*** and Herceptin, respectively. The degree of synergy between two drugs can be quantified by calculating the combination index (CI). CI values <1 indicate synergism, CI values equal to 1 indicate an additive effect, and CI values >1 indicate antagonism.[2]

1.15 *In vivo* evaluation of antitumor activity

SKBR-3 cells were maintained in our lab. In order to build tumor model, we inoculated female Balb/c nude mice with 5×10^6 SKBR-3 cells in the mammary fat pad. Tumor growth was monitored every three days with a caliper over a period of 21 days. The tumors were measured using digital calipers. The tumor volume was calculated by the formula: length×width^2/2. When tumors size reached ~50 mm^3, mice were randomly divided into seven treatment groups with 5 mice in each group and treated with drug dose as shown in Table S1 through the caudal vein. All drugs are injected a total of four times, every three days starting on day 0. Mice weight was also monitored after receiving treatment. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Chinese Academy of Medical Sciences and approved by the Animal Ethics Committee of Animal Experiments and Ethics Review Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (Approval No: IRM-DWLL-2023040).

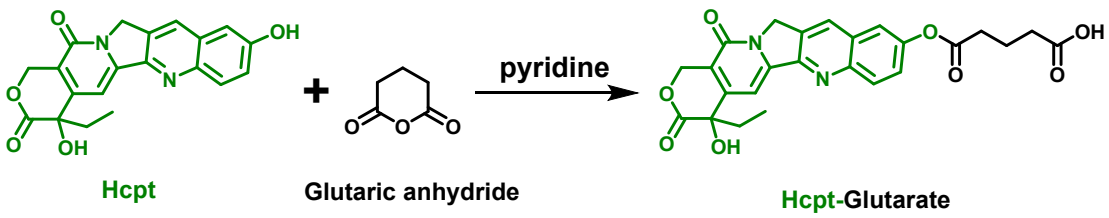
1.16 TUNEL staining

After the experiment, all the mice were euthanized and the tumors were removed and fixed with 4% paraformaldehyde solution, paraffin-embedded, and sectioned. The TUNEL assay was used to determine the level of cell apoptosis in the tumors. Green fluorescence from FITC is indicative of DNA fragmentation in apoptotic cells.

1.17 Statistical analysis

All experiments were independently repeated at least three times under identical experimental conditions. The results were presented as mean ± standard deviations (SD), analyzed with SPSS 25.0 software (IBM Corp., Armonk, NY, USA) and plotted with GraphPad Prism 10 software. Comparison among multiple groups was conducted using one-way analysis of variance (ANOVA) with Bonferroni post-test. Values of $P \leq 0.05$ indicated statistically significant difference. $^*p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

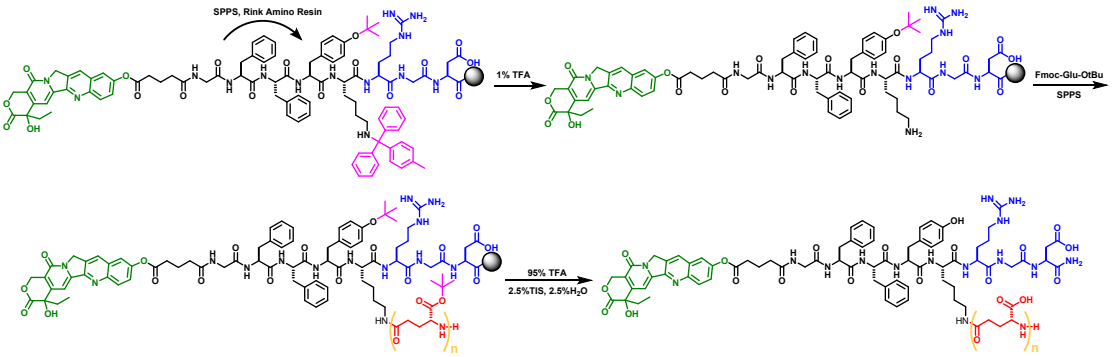
2. Synthesis of Hcpt-Glutaric acid derivative



Scheme S1. The synthetic route for Hcpt-Glutarate.

10-Hydroxycamptothecin (1.092 g, 3 mmol) and Glutaric anhydride (0.353 g, 3.1 mmol) were dissovled in pyridine (30 mL), and the resulting solution was stirred at room temperature overnight. The solution was then removed under reduced pressure to obtained crude product re-dissovled in ethyl acetate (100 ml), and then washed with 1 M HCl (30 ml) to get rid of pyridine for 3 times. The ethyl acetate was removed under vacuum and then add 20 ml H_2O for freeze-drying to get crude product. The product was purified via silica gel column chromatography, eluted with DCM: Methanol (v/v) = 99:1. The brief process was shown in Scheme S1. (89 % yield).

3. Synthesis of Hcpt-GFFYK(γE)$_n$RGD-NH$_2$



Scheme S2. The synthetic route for Hcpt-GFFYK(γE)$_n$RGD-NH$_2$.

The peptide derivative was prepared by solid phase pep tide synthesis (SPPS) using Rink Amide Resin and the corresponding N-Fmoc protected amino acids with side chains properly protected by a tertbutyl group (tBu) or 4 methyl-triphenyl (Mtt).

Piperidine (20%) in anhydrous N,N′-dimethylformamide (DMF) was used during the deprotection of the Fmoc group. 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) was used during the deprotection of the Mtt group. After the last coupling step, excessive reagents were removed by a single DMF wash for 5 min (5 mL per gram of resin), followed by five steps of washing using DCM for 2 min (5 mL per gram of resin). Then 1% TFA was used for the deprotection of the Mtt group for 5 times every 5 min (5 mL per gram of resin). After deprotection of the Mtt group, the resin was washed with DCM and DMF 5 times each. The coupling reagent O-Benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU) and amino acid Fmoc-Glu-OtBu were added to the solid phase synthesis tube. After reacting for 2 hours, the Fmoc protecting group was removed, and another Boc-Glu-OtBu was added to the tube for next amino acid ligation. Lastly, 95% TFA containing 2.5% H_2O and 2.5% TIS was used to cleave peptides derivative from resin and the mixture was filtered. Ice cold diethylether was poured into filtrate concentrated by rotary evaporation. The precipitate was centrifuged for 5 min at a speed of 5000 rpm. The solid was dried by vacuum pump and then purified by HPLC to obtain the pure compounds. The detailed synthetic route and characterizations of the products are shown in Scheme S2 and Figures S1–S4.

4. Supporting results

4.1 Characteristic of compounds

Hcpt-GFFYK(γE)$_2$RGD-NH_2 (Comp. ***1***) : ^{1}H NMR (400 MHz, DMSO) δ 8.49 (s, 1H), 8.28 (t, *J* = 8.1 Hz, 2H), 8.19 (t, *J* = 8.3 Hz, 3H), 8.12 (d, *J* = 9.8 Hz, 1H), 8.05 (dd, *J* = 8.8, 4.2 Hz, 3H), 7.96 (d, *J* = 7.8 Hz, 4H), 7.76 (t, *J* = 6.4 Hz, 1H), 7.50 – 7.42 (m, 2H), 7.35 – 7.29 (m, 4H), 7.25 (d, *J* = 5.4 Hz, 5H), 7.21 – 7.16 (m, 8H), 7.12 (d, *J* = 8.3 Hz, 3H), 6.69 (d, *J* = 8.4 Hz, 2H), 5.44 (s, 2H), 5.26 (s, 2H), 4.60 – 4.43 (m, 6H), 4.30 (dd, *J* = 13.1, 6.3 Hz, 4H), 3.60 – 3.38 (m, 8H), 3.21 – 3.05 (m, 3H), 3.00 (dd, *J* = 21.1, 6.8 Hz, 3H), 2.92 (d, *J* = 8.4 Hz, 4H), 2.87 – 2.75 (m, 5H), 2.73 (s, 1H), 2.71 – 2.63 (m, 2H), 2.63 – 2.54 (m, 2H), 2.44 (s, 1H), 2.22 (t, *J* = 7.5 Hz, 2H), 2.15 (t, *J* = 7.2 Hz, 2H), 1.92 – 1.83 (m, 1H), 1.72 (dd, *J* = 14.7, 7.0 Hz, 4H), 1.65 – 1.47 (m, 7H), 1.46 – 1.40 (m, 1H), 1.36 (d, *J* = 5.7 Hz, 2H), 0.90 (t, *J* = 7.3 Hz, 3H). HR-MS: calc. M = 1705.7049,

obsvd. $((M+2H)/2)^+ = 853.8574$

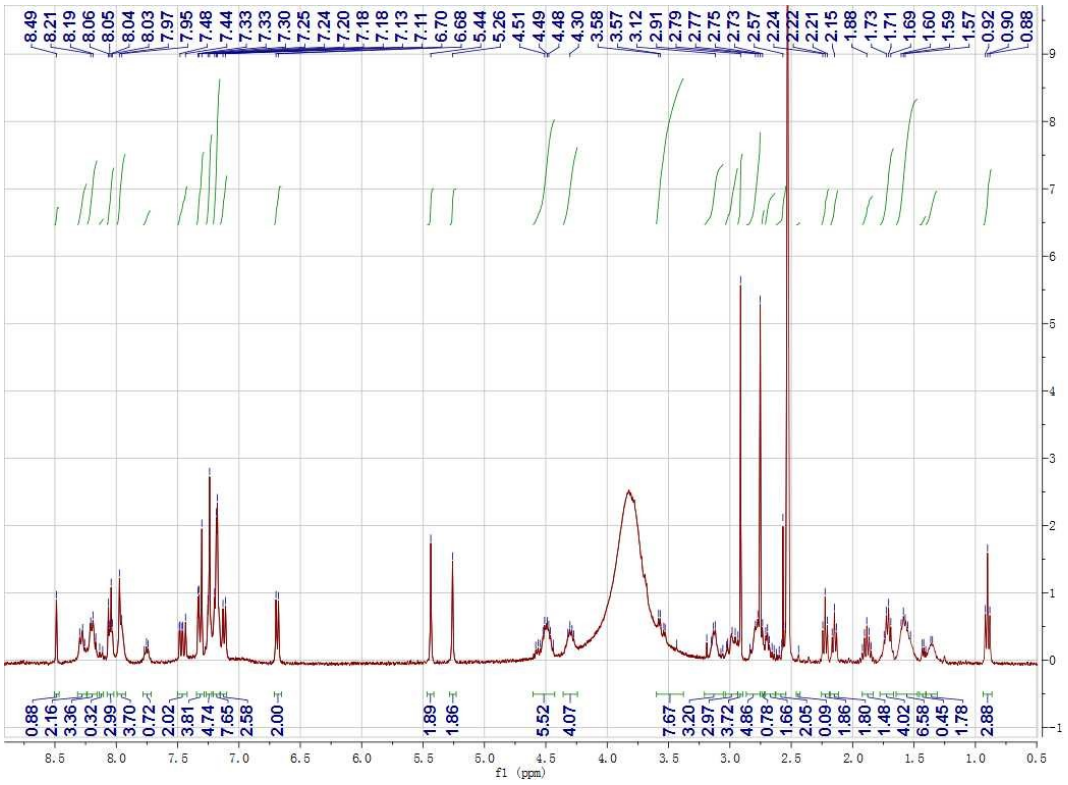


Fig. S1 ^{1}H NMR spectrum (400 MHz, DMSO-d_6, 298 K) of Hcpt-GFFYK(γE)$_2$RGD-NH_2 (**Comp. *1***).

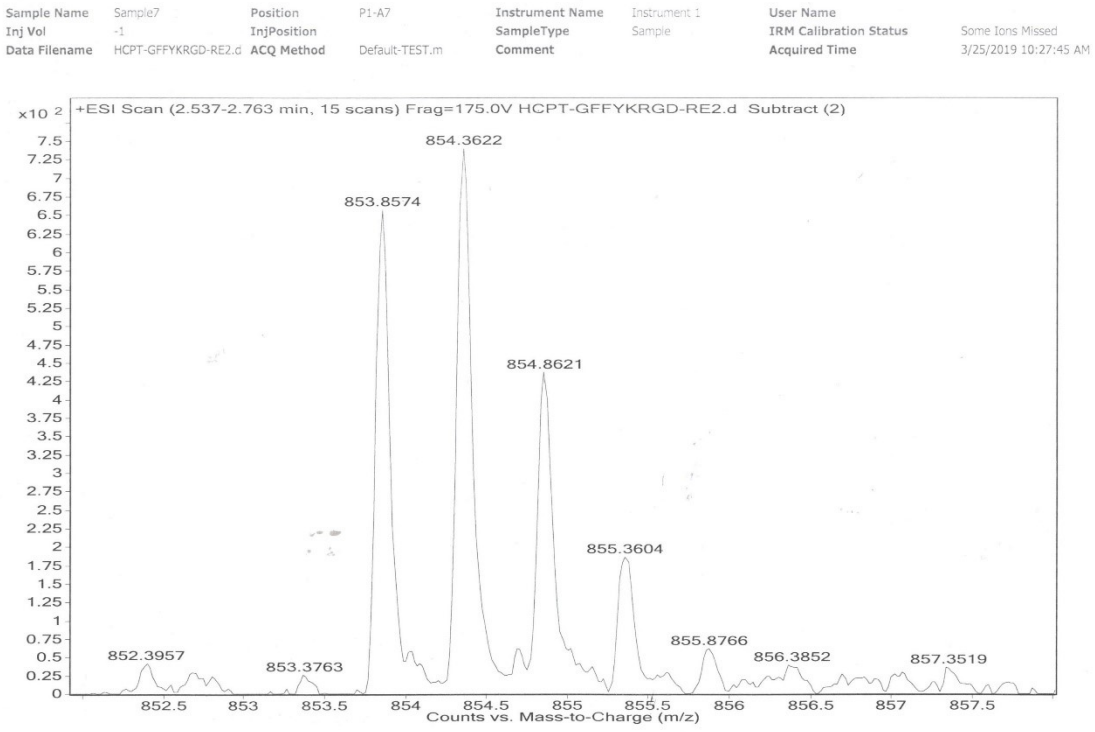


Fig. S2 HR-MS spectrum of Hcpt-GFFYK(γE)$_2$RGD-NH$_2$ (**Comp. *1***). HR-MS: calc. M = 1705.7049, obsvd. ((M+2H)/2)$^+$ = 853.8574

Hcpt-GFFYKRGD-NH$_2$ (**Comp. *2***) : ^{1}H NMR (400 MHz, DMSO) δ 8.70 (s, 1H), 8.49 (s, 1H), 8.43 (s, 1H), 8.25 (s, 2H), 8.19 (s, 4H), 8.06 (d, *J* = 9.1 Hz, 1H), 7.95 (s, 2H), 7.77 – 7.69 (m, 1H), 7.49 – 7.42 (m, 1H), 7.38 (s, 1H), 7.31 (dd, *J* = 12.3, 4.3 Hz, 2H), 7.26 – 7.15 (m, 12H), 7.14 – 7.09 (m, 2H), 6.69 (d, *J* = 8.3 Hz, 2H), 5.45 (d, *J* = 8.7 Hz, 3H), 5.33 (s, 2H), 5.27 (s, 1H), 4.58 – 4.41 (m, 4H), 4.38 – 4.23 (m, 2H), 4.16 (d, *J* = 5.1 Hz, 1H), 3.79 (dt, *J* = 18.3, 6.1 Hz, 3H), 3.62 (d, *J* = 5.7 Hz, 1H), 3.60 – 3.51 (m, 1H), 3.45 (d, *J* = 7.2 Hz, 1H), 3.38 (t, *J* = 7.9 Hz, 1H), 3.31 (dd, *J* = 10.7, 5.7 Hz, 1H), 3.13 (d, *J* = 4.7 Hz, 2H), 3.04 – 2.91 (m, 4H), 2.86 – 2.65 (m, 6H), 2.36 – 2.20 (m, 2H), 2.16 (dd, *J* = 14.6, 7.2 Hz, 1H), 2.10 – 1.99 (m, 1H), 1.99 – 1.83 (m, 3H), 1.80 – 1.65 (m, 3H), 1.64 – 1.46 (m, 6H), 1.37 (ddd, *J* = 10.1, 8.7, 4.6 Hz, 3H), 1.26 (s, 1H), 1.12 – 1.02 (m, 4H), 0.91 (dd, *J* = 9.9, 7.0 Hz, 2H).

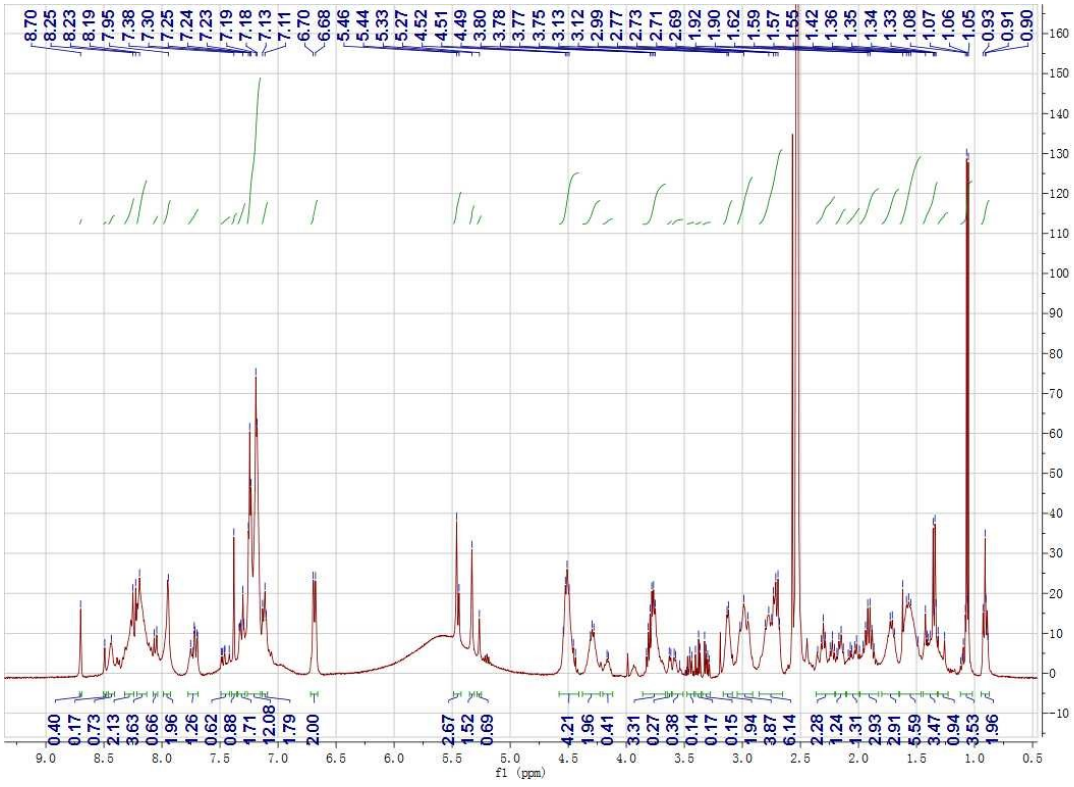


Fig. S3 ^{1}H NMR spectrum (400 MHz, DMSO-d_6, 298 K) of Hcpt-GFFYKRGD-NH$_2$ (**Comp. *2***).

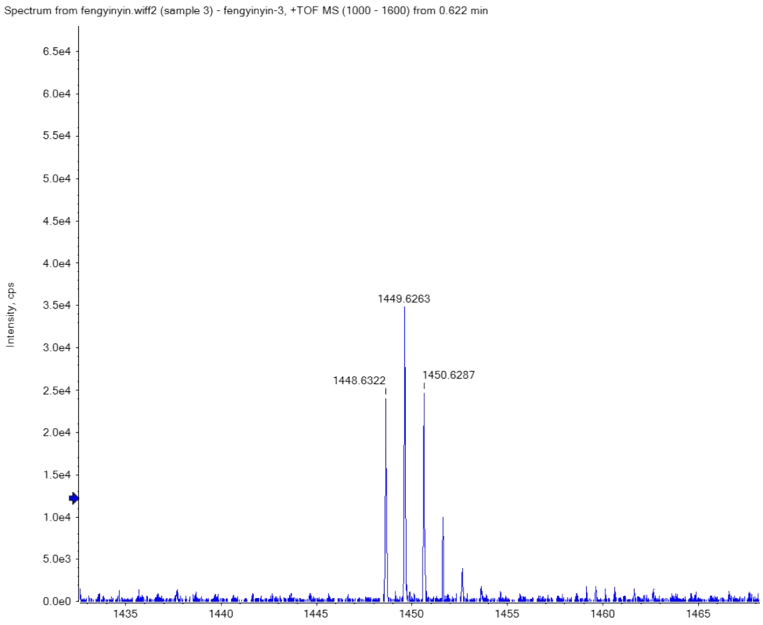


Fig. S4 HR-MS spectrum of Hcpt-GFFYKRGD-NH_2 (**Comp. *2***). HR-MS: calc. M = 1447.6197, obsvd. $(M+H)^+$ = 1448.6322.

4.2 Solubility of *Comp. 1*

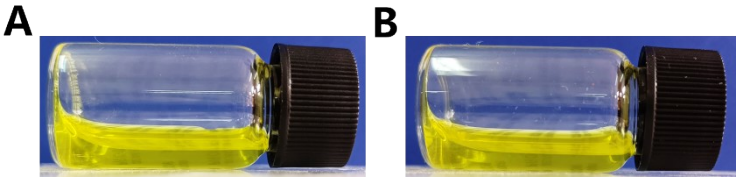


Fig. S5 Optical images of **Comp. *1*** (Hcpt-GFFYK(γE)$_2$RGD-NH_2) at concentrations of (A) 1 wt% and (B) 2 wt%.

4.3 Measurements of Critical aggregation concentration

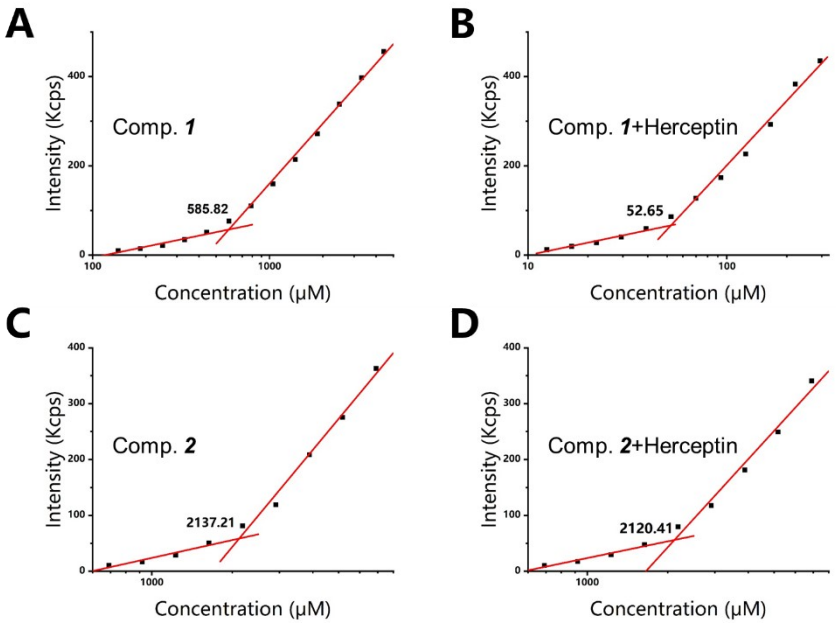


Fig. S6 Critical aggregation concentration (CAC) of (A) **Comp.** ***1***, (B) **Comp.** ***1***+Herceptin, (C) **Comp.** ***2***, and (C) **Comp.** ***2***+Herceptin.

4.4 Measurements of Rheology

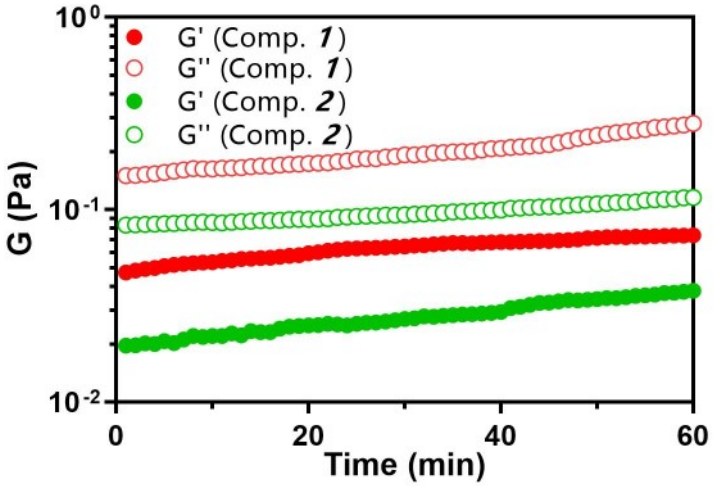


Fig. S7 Dynamic time sweep of solutions formed by **Comp.** ***1*** or **Comp.** ***2***.

4.5 AutoDock

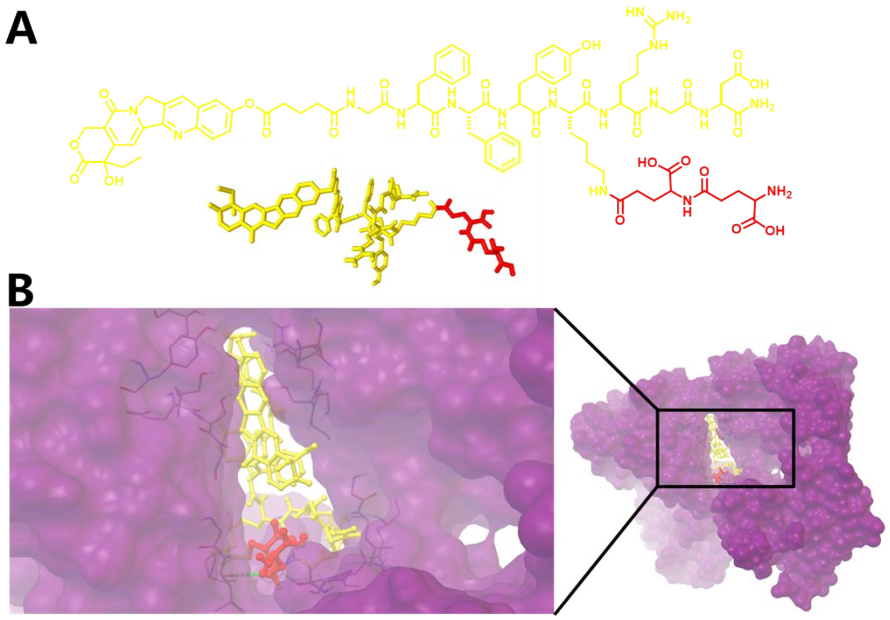


Fig. S8 Detailed interactions between ***Comp. 1*** and Herceptin. (A) The chemical structure of **Comp. *1*** (Hcpt-GFFYK(γE)$_2$RGD-NH$_2$), (B) Stereo view of the interface between ***Comp. 1*** and Herceptin. Herceptin is shown as a purple molecular, ***Comp. 1*** is shown as a yellow and red molecular. Green dotted lines represent hydrogen bonds. The side chains of Herceptin involved in the ***Comp. 1***/Herceptin interaction are depicted as sticks.

4.6 Confocal laser scanning microscopy images

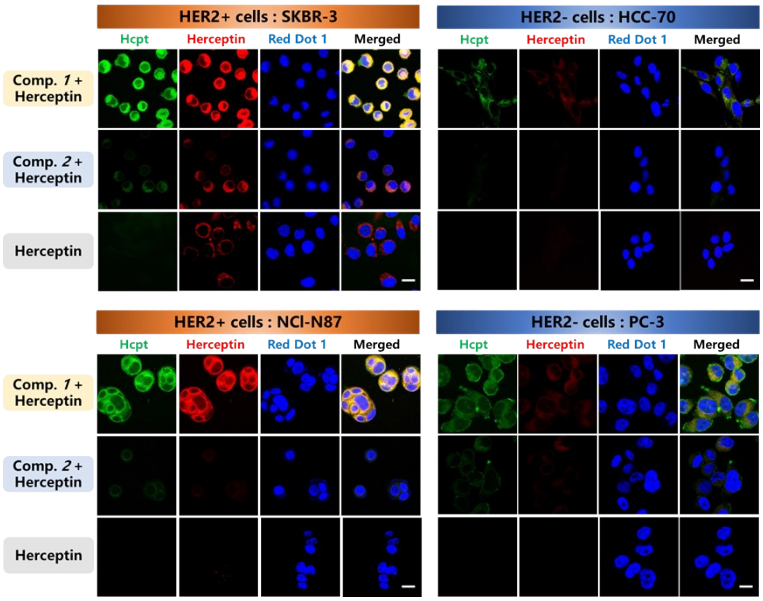


Fig. S9 Confocal laser scanning microscopy images of HER2+ cells (SKBR-3 and NCl-N87) or HER2- cells (HCC-70 and PC-3) incubated with 100 μM of **Comp. *1*** + Herceptin, **Comp. *2*** + Herceptin or Herceptin for 4 h. (scale bars = 25 μm, λ_{exc} = 405 nm for green the channel, λ_{exc} = 488 nm for the red channel, and λ_{exc} = 633 nm for the blue channel).

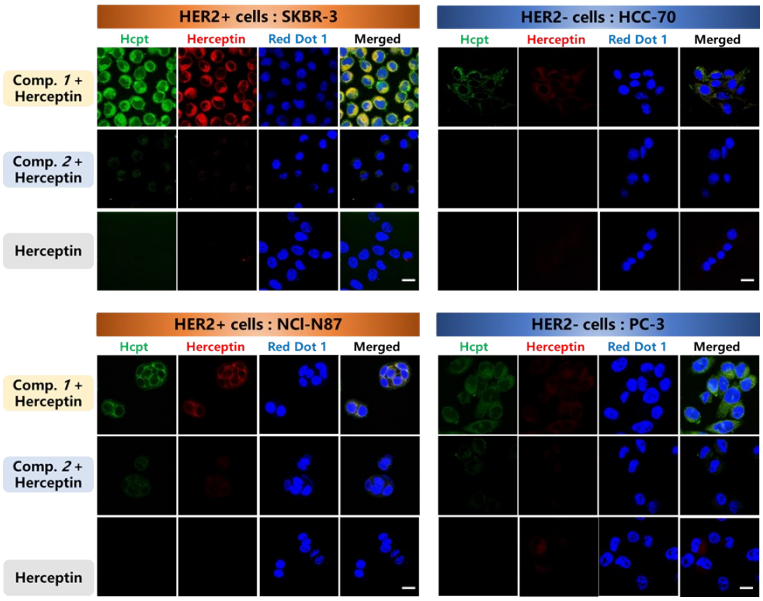


Fig. S10 Confocal laser scanning microscopy images of HER2+ cells (SKBR-3 and NCl-N87) or HER2- cells (HCC-70 and PC-3) incubated with 100 μM of **Comp. *1*** +Herceptin, **Comp. *2*** + Herceptin or Herceptin for 1 h. (scale bars = 25 μm, λ_{exc} = 405

nm for green the channel, λ_{exc} = 488 nm for the red channel, and λ_{exc} = 633 nm for the blue channel).

4.7 Cell inhibition analysis

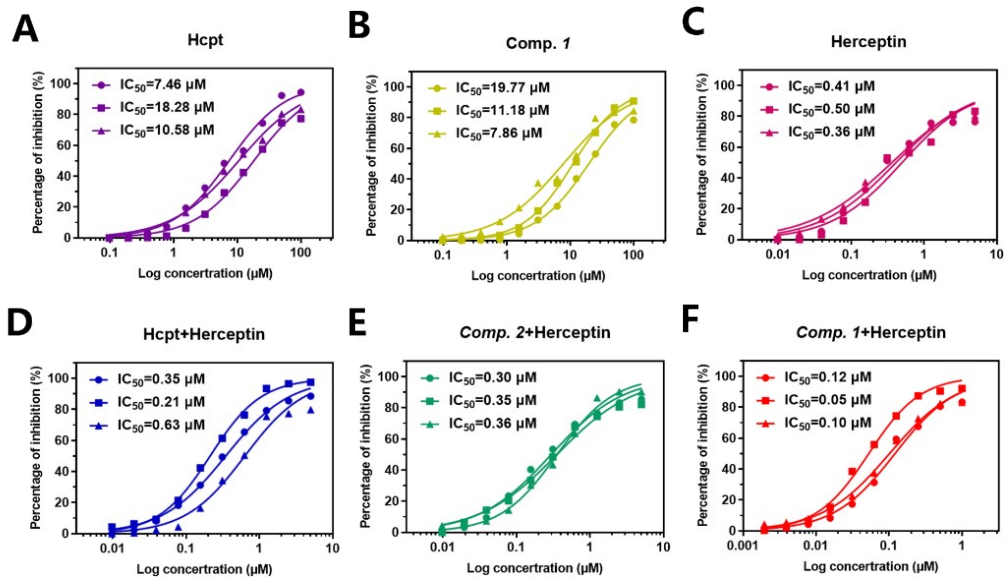


Fig. S11 IC_{50} profiles of different drugs against SKBR-3 cancer cells.

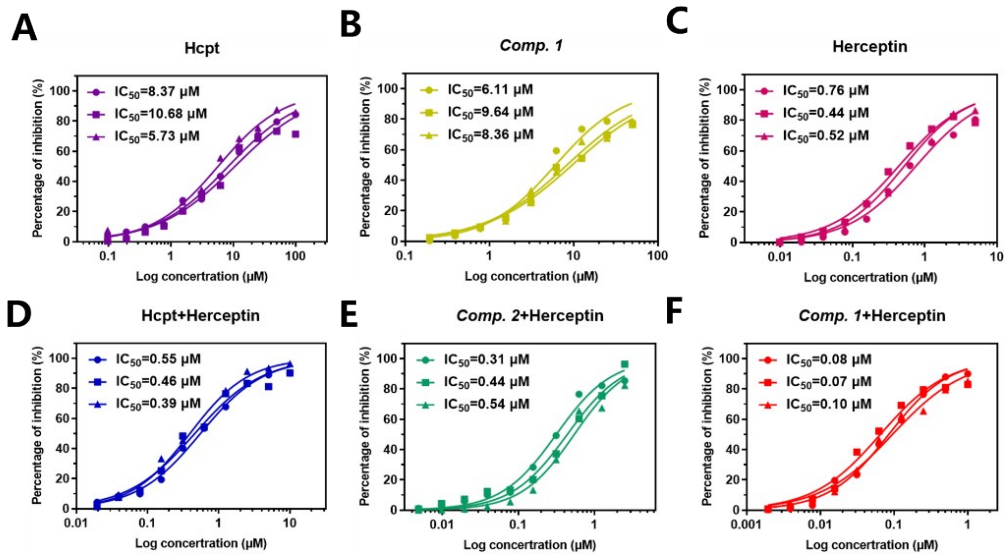


Fig. S12 IC_{50} profiles of different drugs against NCl-N87 cancer cells.

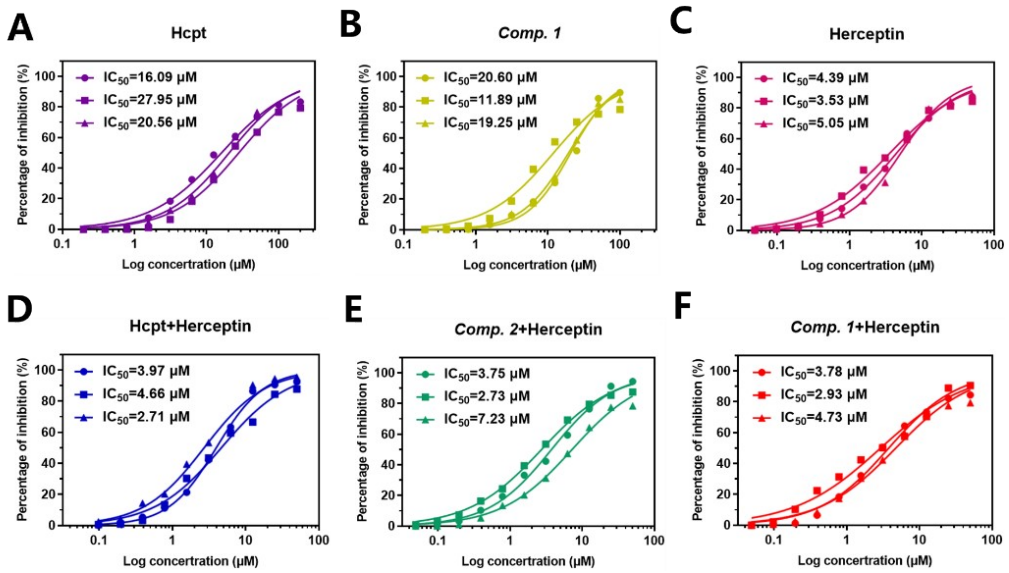


Fig. S13 IC_{50} profiles of different drugs against HCC-70 cancer cells.

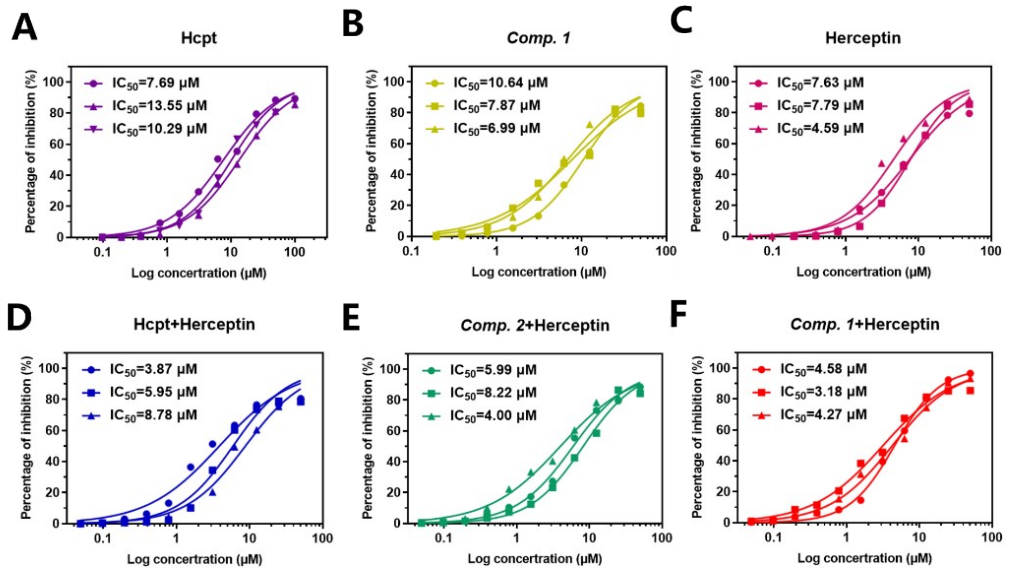


Fig. S14 IC_{50} profiles of different drugs against PC-3 cancer cells.

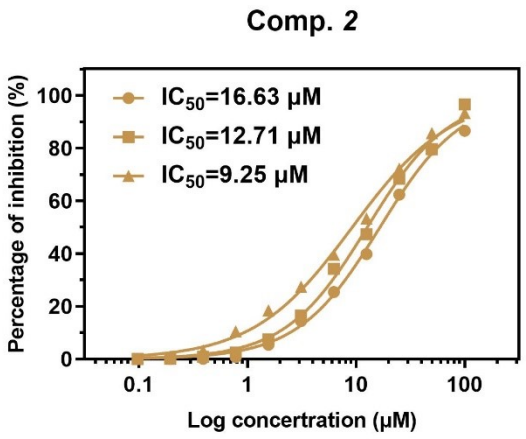


Fig. S15 IC_{50} profiles of ***Comp. 2*** against SKBR-3 cancer cells.

4.8 Drug formulations and doses

Group	Drug	Dose
1	PBS	0.90% saline
2	Hcpt	1.50 mg/kg
3	Herceptin	2.00 mg/kg
4	Comp. ***1***	7.02 mg/kg (content of HCPT equal to group 2)
5	Hcpt+Herceptin	1.50 mg/kg+2.00 mg/kg
6	Comp. ***2***+Herceptin	5.96 mg/kg+2.00 mg/kg (content of HCPT equal to group 2)
7	Comp. ***1***+Herceptin	7.02 mg/kg+2.00 mg/kg (content of HCPT equal to group 2)

Table S1. Drug formulations and doses for evaluation of tumor inhibition.

4.9 Tumor growth curves

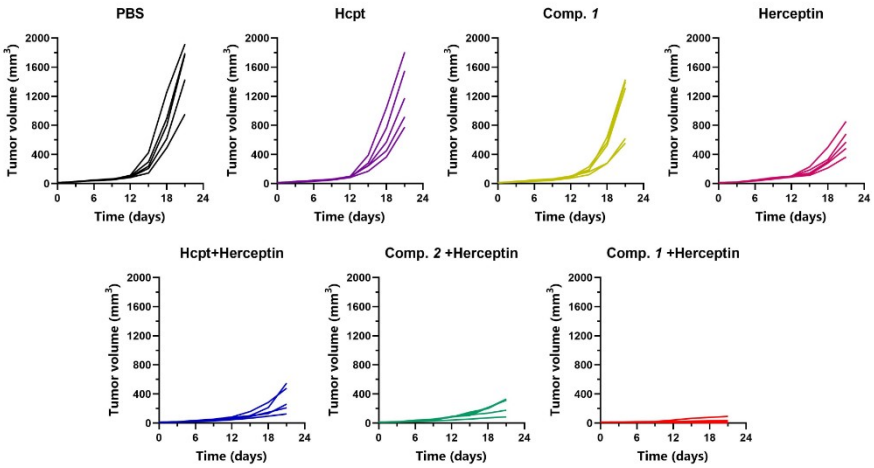


Fig. S16 Tumor growth curves for each mouse in the different groups.

4.10 Body weight of SKBR-3 tumor-bearing mice

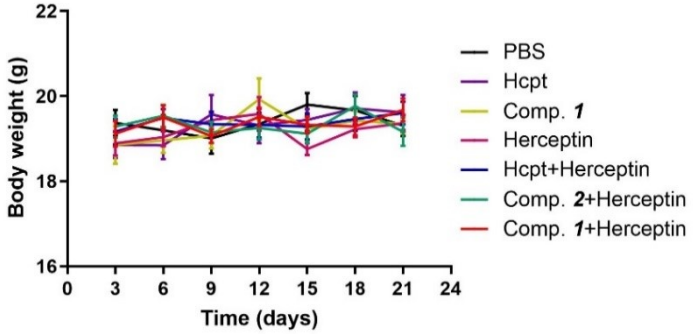


Fig. S17 Body weight of SKBR-3 tumor-bearing mice was monitored every three days. The data are shown as mean ± SEM (n = 5).

5. References

[1] O. Trott, A. J. Olson, *J. Comput. Chem.* 2010*,* **31***, 455.*

[2] Y. Cai, H. Shen, J. Zhan, M. Lin, L. Dai, C. Ren, Y. Shi, J. Liu, J. Gao, Z. Yang, *J. Am. Chem. Soc.*, 2017, **139**, 2876.