Enzyme-triggered supramolecular self-assemblies of Pt prodrug for site-specific drug accumulation and enhanced antitumor efficacy

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Supporting information

Materials and methods

Cis-$Pt(NH_3)_2Cl_2$ was supplied from Sigma-aldrich.. Phenylalanine, lysine, tyrosine amino acid was obtained from Shanghai GL Biochem. Alkaline phosphatase was purchased from Fermentas Life Science. (Unit Size: 300 units, 1.0 unit per μL). All the other starting chemical reagents and solvents were received from Sigma-aldrich. and J&K Chemical without further purification unless otherwise noted. ^1H and ^{31}P NMR characterizations were performed on a Varian Unity Inova 400. Atomic force microscopy (AFM) and Transmission electron microscopy (TEM) images were obtained on a Bruker multimode 8 system with the tip of Bruker scanasyst-air and Tecnai G220 transmission electron microscope respectively. LC-MS analyses were performed on an Agilent 6120 Quadrupole LC/MS system with an ESI resource. HPLC purification and analysis were carried out on a Waters 600E Multi-solvent Delivery System using a YMC C18 RP column with CH_3CN (0.1% of TFA) and water (0.1% of TFA) as the eluents. Fluorescence spectroscopy measurements were recorded by Thermo Scientific Varioskan Flash spectrak scanning multimode reader. CD spectra were recorded on an Aviv Model 410. Fluorescence confocal images were taken on a Leica TCS SP5 II confocal laser scanning microscope. Platinum contents were measured with an inductively coupled plasma-mass spectrometry (ICP-MS Varian 710-ES).

Synthesis and Characterizations

Solid-phase peptide synthesis (SPPS) of 5

Compound 5 was prepared by the standard solid-phase peptide synthesis by using 2-chlorotrityl chloride resin and different *N*-Fmoc protected amino acids with side chains properly protected. Fmoc-Tyr(PO_3H_2)-OH was prepared from L-Tyr-OH by following the reaction conditions and procedures from previous study,[1] and was used directly in solid-phase peptide synthesis. The resin was firstly swelled in dry dichloromethane for 30 minutes, followed by thoroughly washing with dry *N*,*N*-

dimethylformamide (DMF) for three times. Then the first amino acid Fmoc-Tyr(PO_3H_2)-OH in DMF solution with the presence of *N*,*N*-diisopropylethylamine was loaded onto resin, and reacted for 1 hour. After washed with DMF, the unreacted sites in resin were quenched by using blocking solution (80: 15: 5 of DCM/MeOH/DIPEA) for 2 x 10 minutes. Then the resins were treated with 20% piperidine (in DMF) for 0.5 hour to remove the protecting group, followed by washing the resin in DMF for five times. Then we conjugated the subsequent Fmoc-protected amino acid to the free amino group on the resin using HBTU as the coupling reagent. These coupling and deprotection steps were repeated to elongate the peptide chain, which were carried out by following the standard Fmoc solid-phase peptide synthesis protocol. Finally, the resin was washed with DMF (5 times), DCM (5 times), methanol (5 times), and hexane (5 times) respectively, then the peptide was cleaved from the resin by using trifluoroacetic acid for 3 hours. The resulted crude products were purified by using HPLC with water-acetonitrile as eluent (from 80: 20 to 0: 100) to afford the final product (yield: 43%).^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.33 (d, 1H), 8.19 (d, 1H), 8.09 (d, 1H), 7.97 (s, 3H), 7.84 (d, 1H), 7.73 (d, 1H), 7.70 (d, 1H), 7.67 (d, 1H), 7.59 (s, 1H), 7.47-7.38 (m, 2H), 7.28-7.05 (m, 15H), 4.58-4.43 (m, 2H), 4.43-4.36 (m, 1H), 4.19 (s, 1H), 3.60-3.48 (dd, 2H), 3.09-2.95 (m, 2H), 2.93-2.84 (m, 2H), 2.78 (dd, 1H), 2.67 (dd, 1H), 2.51 (s, 2H), 1.41-0.91 (m, 6H). ^{13}C NMR (400 MHz, DMSO-d_6) δ: 173.25, 171.56, 171.42, 170.94, 170.21,138.20, 138.15, 134.32, 133.33, 132.13, 130.64, 130.03, 129.67, 129.64, 129.27, 128.03, 127.67, 127.65,127.24, 126.63, 126.55, 126.38, 125.86, 119.60, 54.35, 54.08,53.53, 53.29, 42.67, 38.93, 37.96, 37.61, 35.85, 32.41, 27.03, 22.42. ^{31}P NMR (400 MHz, DMSO-d_6) δ: 6.03. MS: calcd M=851.33; obsd $(M-H)^-$=850.3.

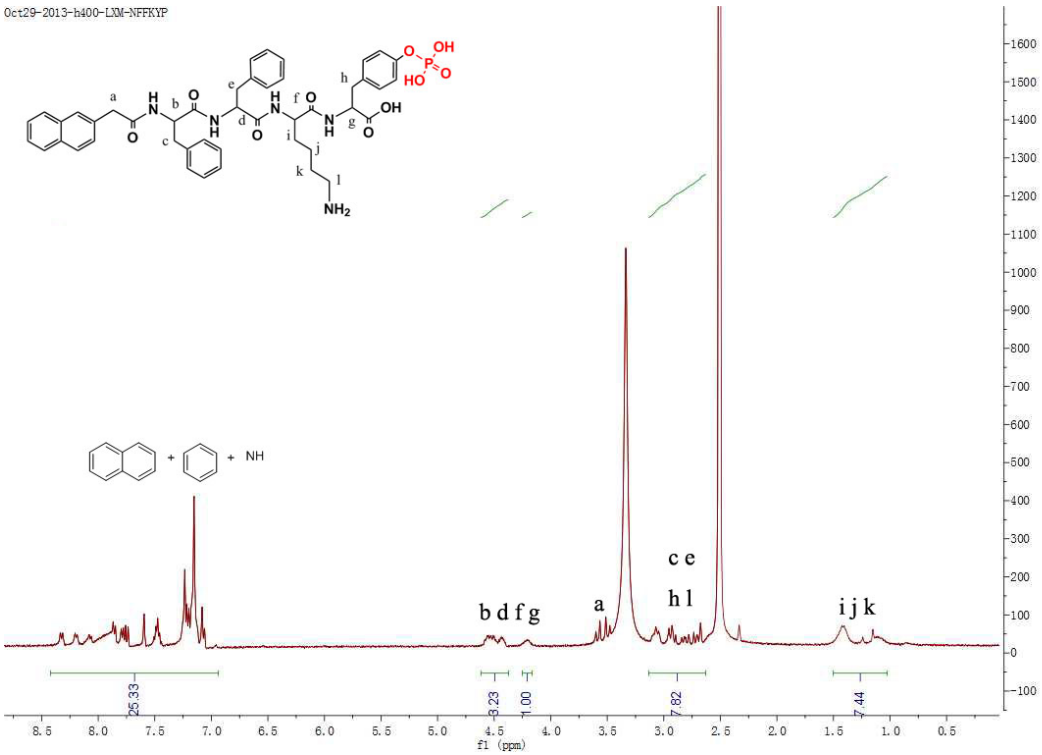


Figure S1. ^{1}H NMR spectrum of **5** in DMSO-d_6.

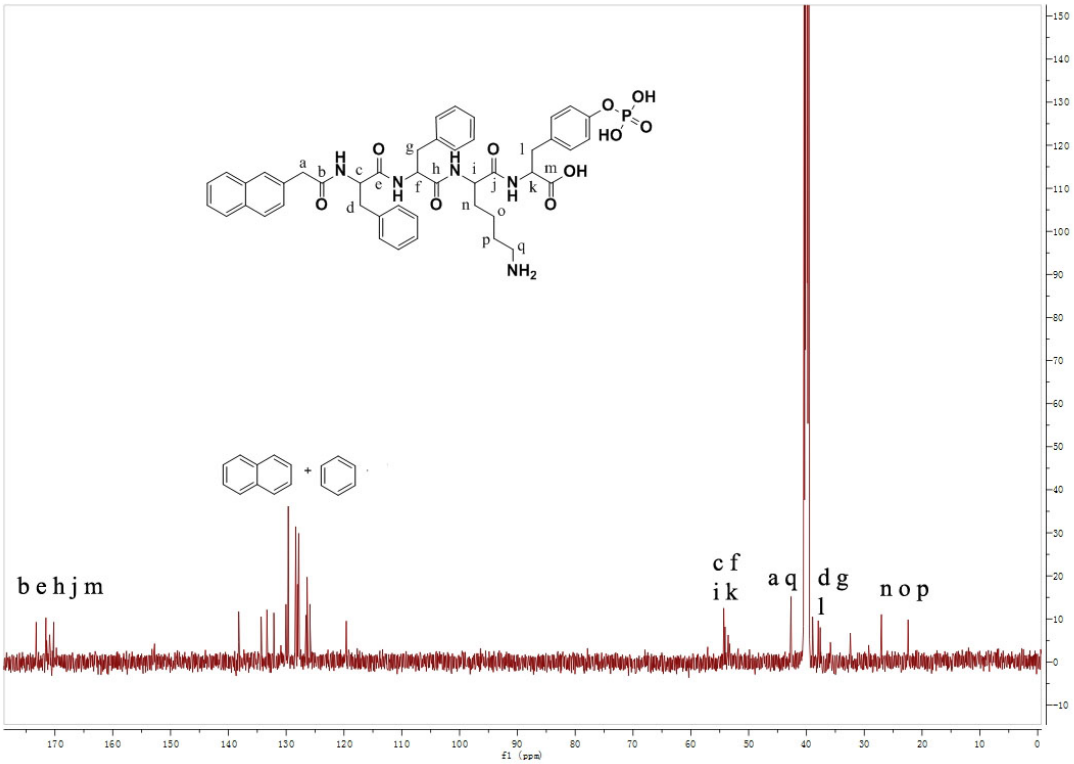


Figure S2. ^{13}C NMR spectrum of **5** in DMSO-d_6.

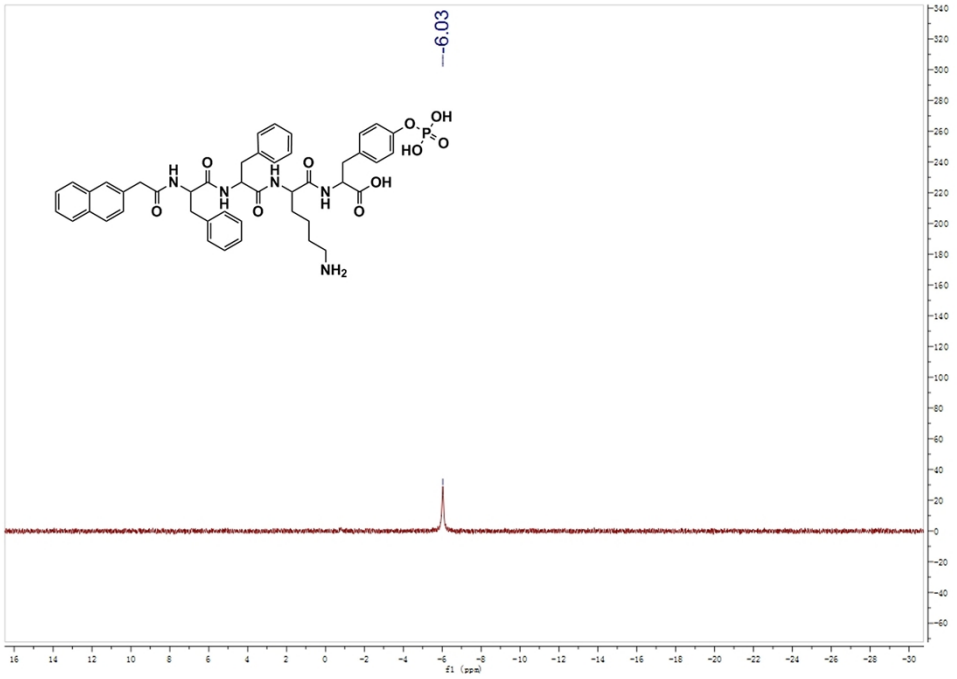
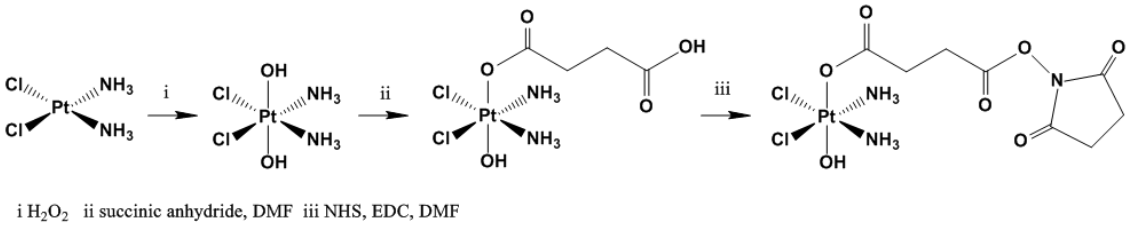


Figure S3. ^{31}P NMR spectrum of **5** in DMSO-d_6.

Synthesis of cisplatin (IV) prodrug.



Scheme S1. The synthetic route for the preparation of **4**.

Synthesis of 3

The Pt (IV) compound was synthesized by following the typical reaction procedures.[2] *Cis*-$Pt(NH_3)_2Cl_2$ (400 mg, 1.33 mmol) and H_2O_2 (30 wt%, 22.74 mL, 200.0 mmol) were dissolved in H_2O (18 mL) and heated at 70 ℃ with vigorous stirring for 5 h in the dark. After concentrated by vacuum, *c,c,t*-$Pt(NH_3)_2Cl_2(OH)_2$ (355 mg, 80%) was obtained from filtration. To the solution of *c,c,t*-$Pt(NH_3)_2Cl_2(OH)_2$ (200 mg, 0.6 mmol) in DMF (10 mL) was added succinic anhydride (60 mg, 0.6 mmol) and the mixture was stirred for 6 h at 75 ℃. The resulting solution was dried to dark yellow oil under vacuum, followed by the addition of a small volume of acetone. After precipitated by ether, the pale yellow product (yield: 57%) was isolated via vacuum. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.50 (br, 6H), 2.4-2.3 (m, 4H). ^{13}C NMR (400 MHz, DMSO-d_6) δ: 180.04, 174.25, 30.90, 30.32. ^{195}Pt NMR (400 MHz, DMSO-d_6) δ: 1228.24. MS: calcd M=434.14; obsd $(M-H)^-$=433.0.

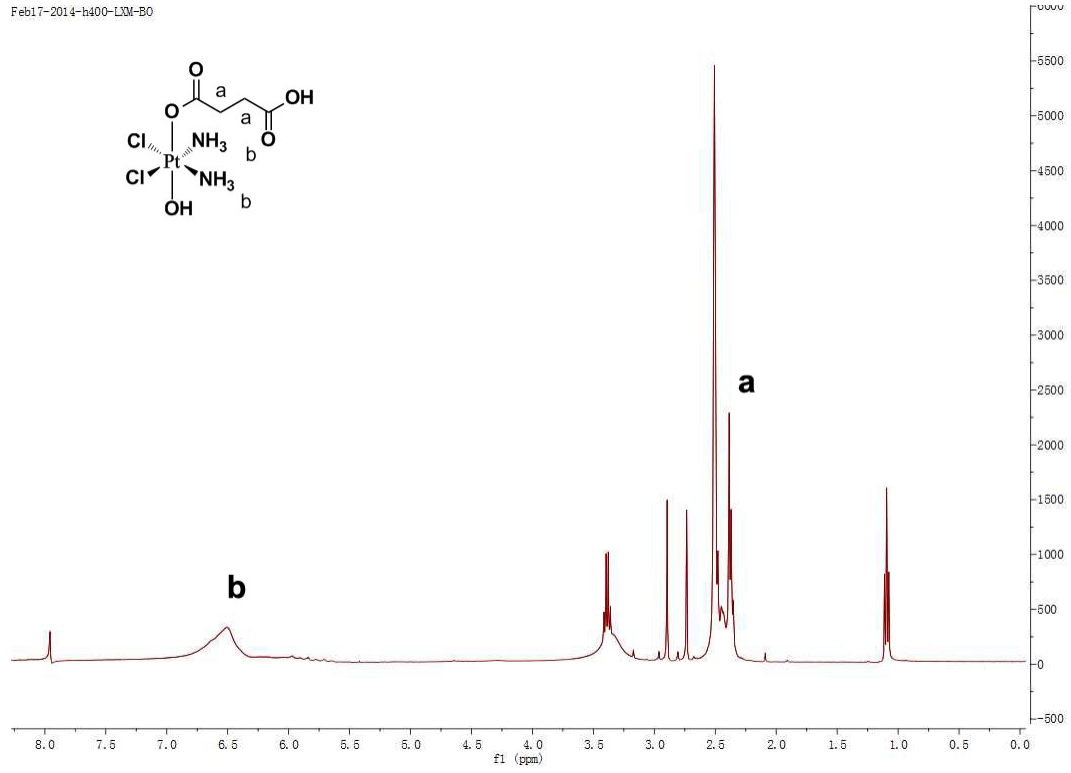


Figure S4. ^{1}H NMR spectrum of **3** in DMSO-d_6.

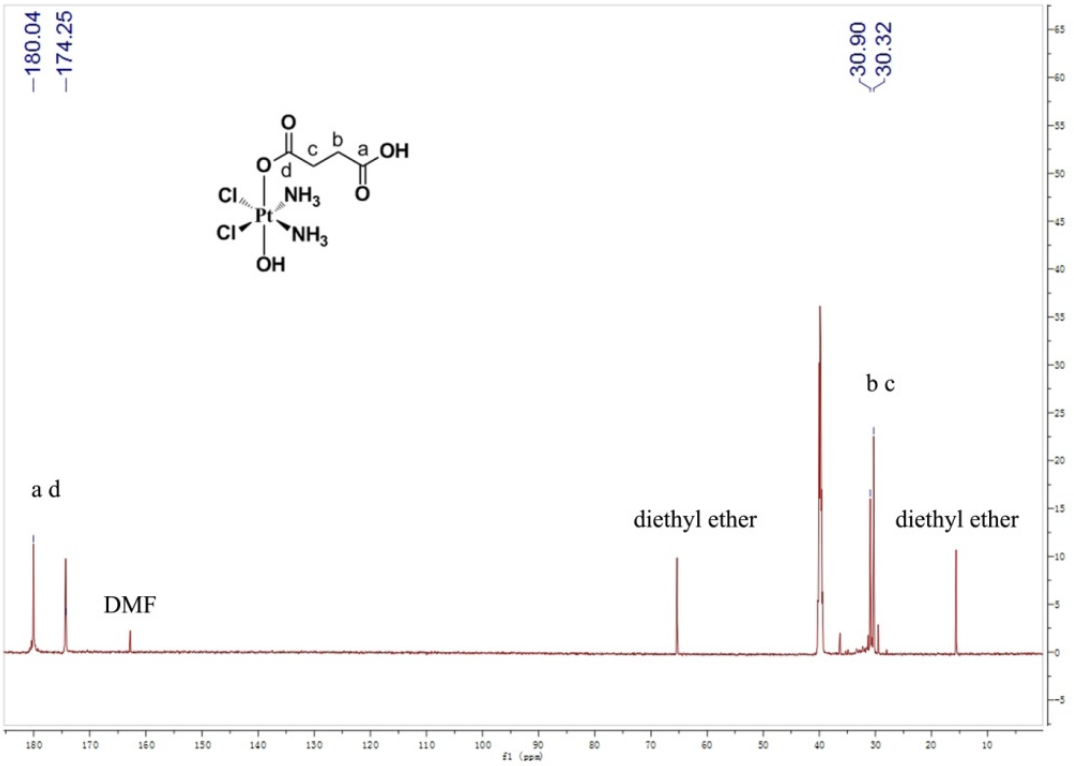


Figure S5. ^{13}C NMR spectrum of **3** in DMSO-d_6.

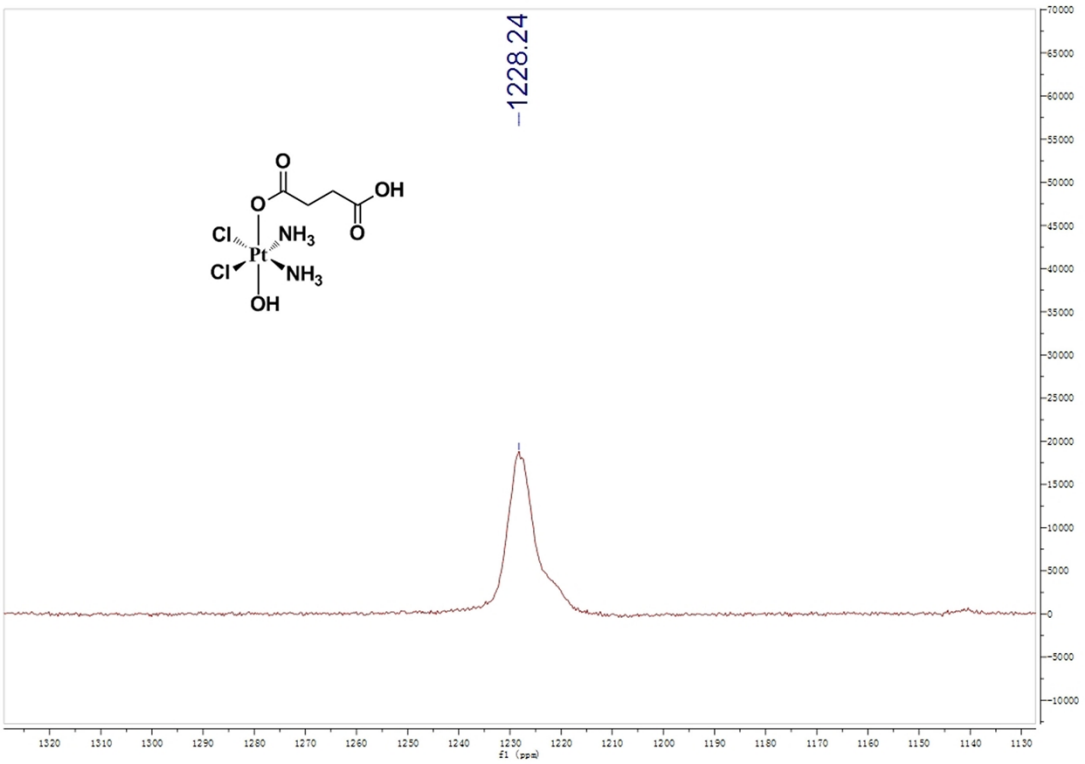


Figure S6. ^{195}Pt NMR spectrum of **3** in DMSO-d_6.

Synthesis of 4

3 (130 mg, 0.3 mmol) was reacted with *N*-hydroxysuccinimide (51.7 mg, 0.45 mmol) and EDC/HCl (86.3 mg, 0.45 mmol) in anhydrous DMF (10 mL) for 12 h at room temperature. The mixture was then purified by HPLC with water-acetonitrile as eluent (from 100: 0 to 0: 100). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.50 (br, 6H), 2.81 (s, 4H), 2.63-2.59 (m, 4H). MS: calcd M=530.21; obsd $(M+H)^{-}$=531.8.

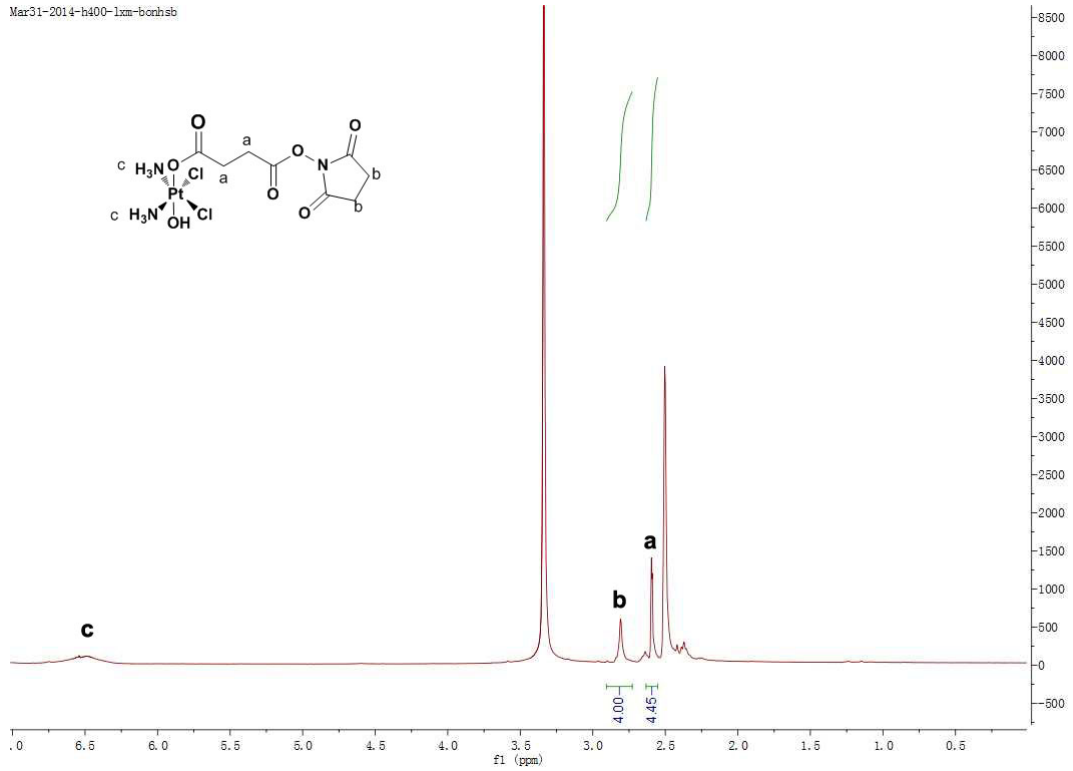


Figure S7. ^{1}H NMR spectrum of **4** in DMSO-d_6.

Synthesis of Pt (IV)-based precursor 1

Compound **5** (42.6 mg, 0.05 mmol) and DIPEA (30 μL) were dissolved in anhydrous DMF (10 mL), and stirred at room temperature for 10 min. Then **4** (53.0 mg, 0.1 mmol) was added to the mixture and reacted at room temperature for 12 h. The resulting solution was purified by using HPLC with water-acetonitrile as eluent (from 80: 20 to 0: 100) to give product **1** (yield: 27%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.35 (d, 1H), 8.22 (d, 1H), 8.08 (d, 1H), 8.01 (s, 3H), 7.93 (d, 1H), 7.84 (d, 1H), 7.78-7.73(m, 3H), 7.59 (d, 1H), 7.46 (d, 1H), 7.24-7.09 (m, 15H), 6.56 (s, 6H) 4.52-4.46 (m, 2H), 4.46-4.39 (m, 1H), 4.14 (s, 1H), 3.56-3.49 (dd, 1H), 3.07-2.65 (m, 10H), 2.38-2.27 (m, 2H), 1.56-1.15 (m, 6H). ^{13}C NMR (400 MHz, DMSO-d_6) δ: 174.34, 173.26, 171.98, 171.12, 170.24, 158.59, 158.38,138.21, 138.13, 134.31, 133.32, 131.40, 129.63, 127.65, 127.64, 126.54, 126.37, 125.85, 120.50, 120,19, 118.52, 116.47, 114.55, 72.92, 63.50, 54.28, 52.82, 42.61, 38.95, 37.75, 36.42, 34.64, 32.44, 30.48, 29.71, 29.33, 23.04. ^{195}Pt NMR (400 MHz, DMSO-d_6) δ: 1231.20.

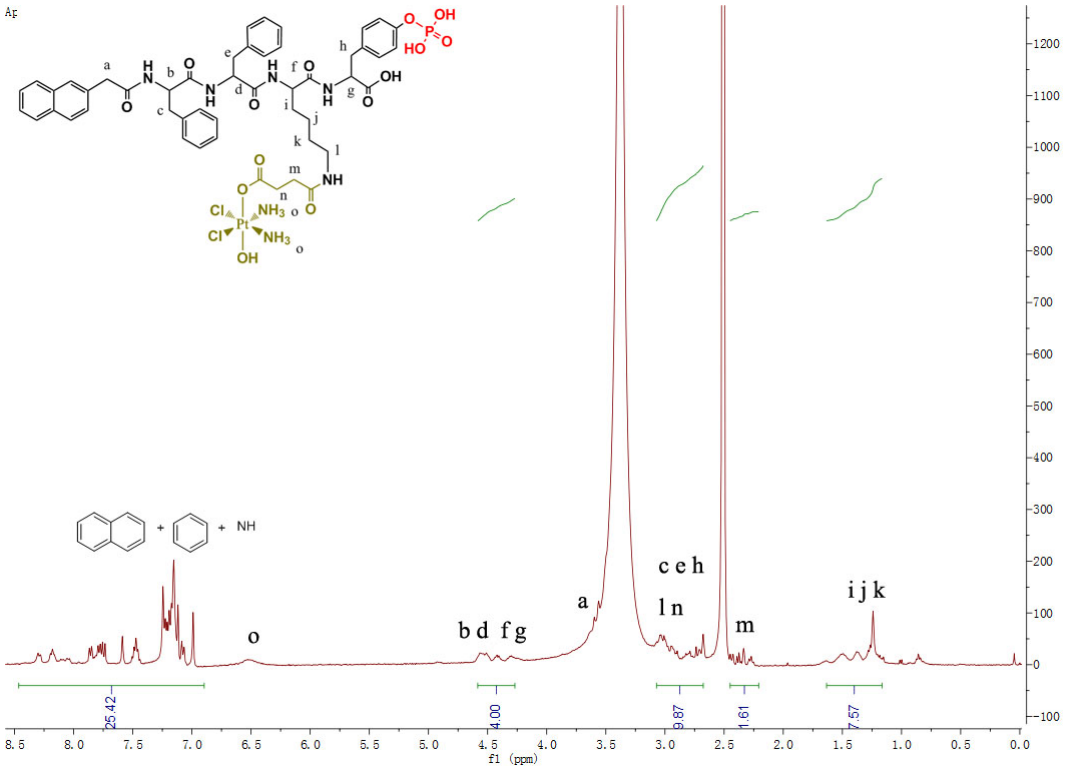


Figure S8. ^{1}H NMR spectrum of prodrug **1** in DMSO-d_6.

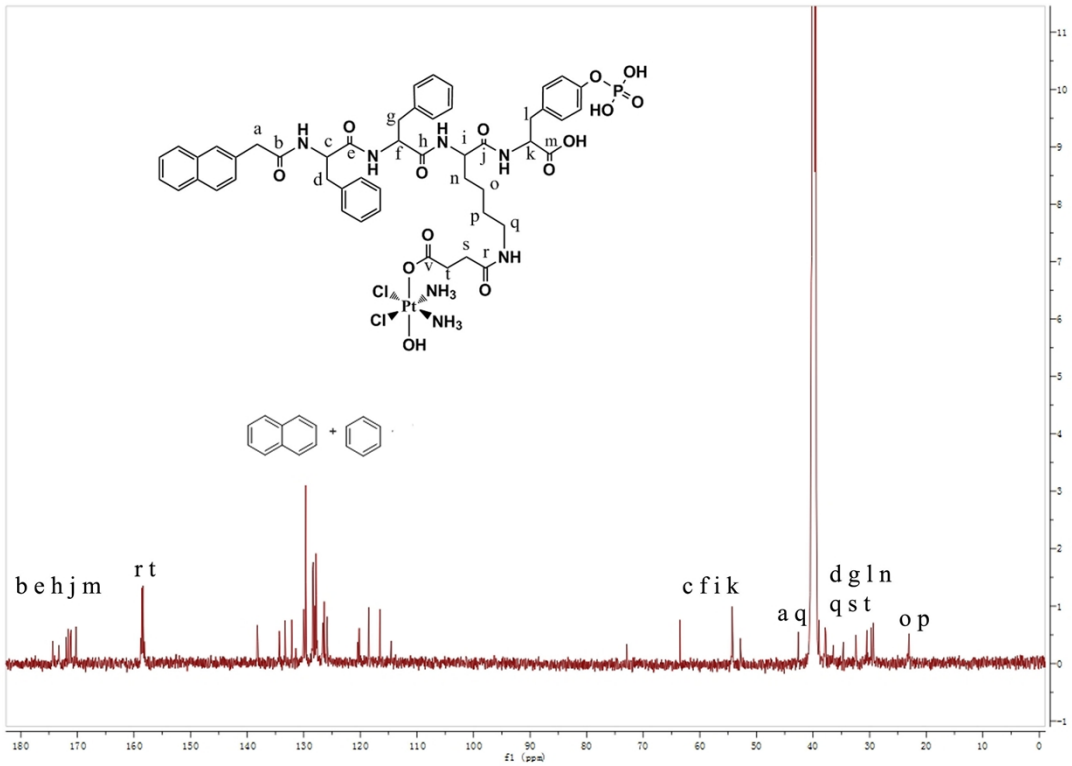


Figure S9. ^{13}C NMR spectrum of prodrug **1** in DMSO-d_6.

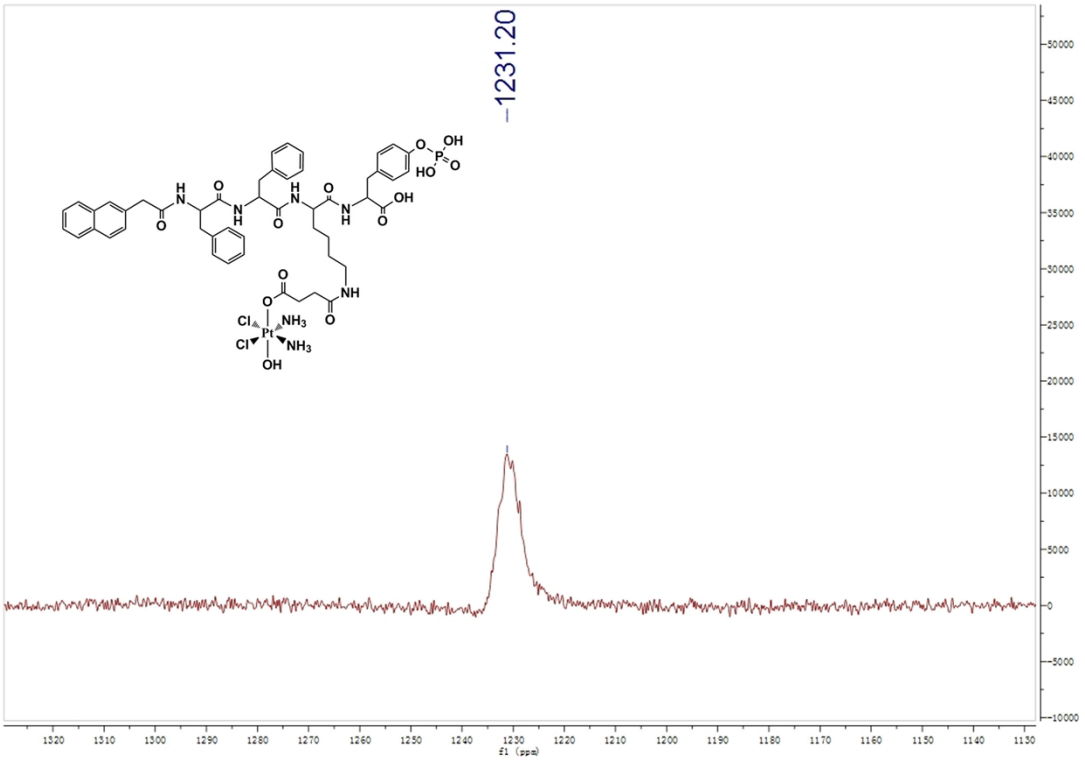
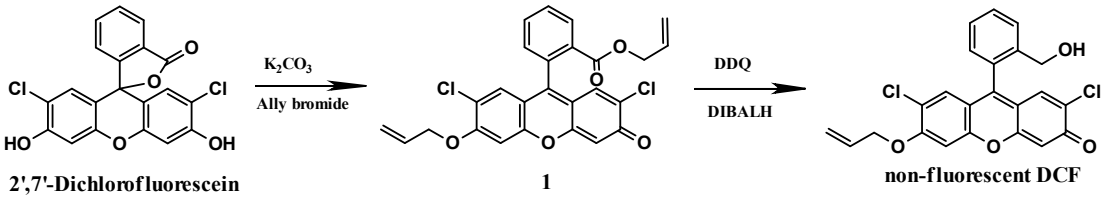


Figure S10. ^{195}Pt NMR spectrum of prodrug **1** in DMSO-d_6.

Synthesis of non-fluorescent DCF



Scheme S2. The synthesis procedure of non-fluorescent DCF.[3]

2', 7'-Dichlorofluorescein (100 mg, 0.25mmol), K_2CO_3 (100 mg, 0.75 mmol) and allyl bromide (0.065 ml, 0.75 mmol) were added to DMF (2 mL), and the mixture was stirred continuously for 2.5 h at room temperature. Then cold ice water was added and extracted with ethyl acetate. The organic layers were dried by magnesium sulfate, filtered and dried in vacuum to give compound **6**. To a solution of compound **6** (50 mg) in dichloromethane (1 mL) was added diisobutyl aluminium hydride (DIBALH) (0.5 mL, 1.0 M in hexanes) dropwise at -78℃ over 10 min under N_2 protection, and then the solution was allowed to warm to room temperature and stirred for 2 h. Then the reaction mixture was cooled to 0℃, followed by the addition of diethyl ether (3 mL) and saturated NH_4Cl (0.5 mL). This mixture was warmed to room temperature and stirred for another 1 h. Finally, 5 mL of 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (26.2 mg) solution was added at 0℃, and stirred for 1 h at room temperature. This final product of non-fluorescent DCF was purified by silica gel

chromatography (Yield: 38%). ^{1}H-NMR (400 MHZ, DMSO-d_6): δ 7.50-7.46 (m, 2H), 7.42-7.39 (m, 1H), 7.30-7.27 (m, 1H), 7.08 (s, 1H), 6.91 (s, 1H), 6.85 (s, 1H), 6.81 (s, 1H), 6.08 (m, 1H), 5.48 (m, 1H), 5.44 (m, 1H), 5.34 (s, 2H), 4.73 (m, 2H). MS: calcd M=426.04; obsd $(M-H)^-$=425.0.

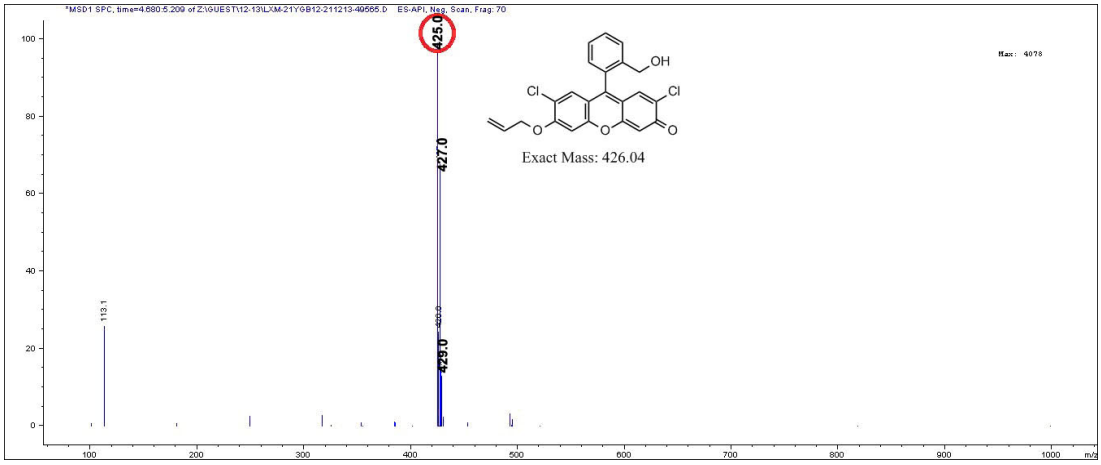


Figure S11. MS spectra analysis for the generation of non-fluorescent DCF.

Procedure for hydrogel preparation

2 units of alkaline phosphatase were added to the prodrug **1** solution (1.0 wt%) in PBS buffer (10 mM, pH 7.4), and the mixture solution was left on bench to allow for hydrogelation in 2 hr.

Rheological measurements

Rheological tests were conducted on Thermo Scientific HAAKE RheoStress 6000 rheometer with a 20 mm parallel plate. 0.2 mL of the hydrogel sample was placed on the cone-plate. The dynamic strain sweep test was run from 0.1 to 10% strain with frequency at 6.282 rad s^{-1} at 25 ℃. The dynamic frequency sweep test was run from 200 rad/s to 0.1 rad/s and a strain at 1.0% was used to ensure the linearity of dynamic viscoelasticity.

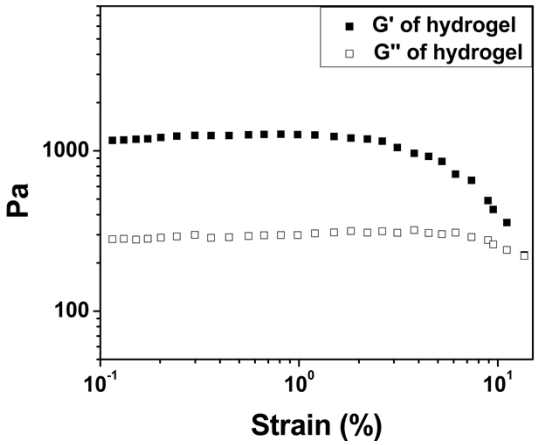


Figure S12. Strain dependence of the dynamic storage moduli (G') and the loss moduli (G") of the hydrogel shown in Fig. 1A.

Circular Dichroism

CD spectra were recorded from 185 to 400 nm by using an Aviv 410 spectrometer under a nitrogen atmosphere. The hydrogels (0.1 ml, 1.0 wt%) or solution was placed evenly on the 1 mm thick quartz curvet and scanned with 0.5 nm interval.

Fluorescence-based quantification of platinum

A 4 mM solution of non-fluorescent dichlorofluorescein and a 40 mM solution of PPh3 were prepared by using DMSO as solvent. A varying and known concentrations of cisplatin solutions were prepared in 20: 80 DMSO/PBS (pH=7.4), which are used as standard for calibration studies. After mixing above solutions and incubated for 3 h at room temperature in the dark, the fluorescence intensities of the mixtures were recorded by microplate reader, and then a standard curve about the fluorescent intensities of the solutions and Pt(II) concentrations was obtained. The released Pt(II) from hydrogel in PBS solution was quantified by this standard curve.

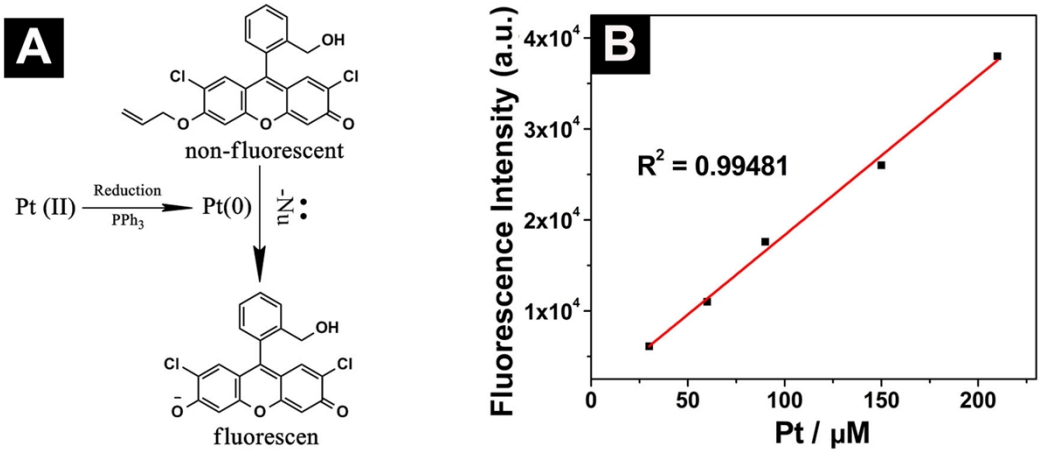


Figure S13. (A) The mechanism for fluorescence turn-on of dichlorofluorescein triggered by Pt(II) complex through the allyl ether cleavage; (B) the standard curve

between Pt(II) concentrations and fluorescence intensities..

Drug release study

0.2 mL of fresh PBS buffer solution (10 mM, pH=7.4) containing different concentrations of glutathione (GSH) (0-5 mM) was added onto the top of the gel. At different time-scale, the 0.2 mL PBS buffer was taken out for analysis, and another 0.2 mL of same fresh PBS buffer was added onto the top of the hydrogels. The amount of Pt(II) in PBS solution was determined by the method of fluorescence-based quantification of Pt(II) drug release and ICP-OES.

Table S1. ICP-OES data for drug release.

Time(h)	Concentration(Pt)	Sum(Pt)	%
5 mM GSH			
0	0 mg/L	0 mg/L	0
0.5	3.1 mg/L	3.1 mg/L	4.0
1	4.0 mg/L	7.1 mg/L	9.2
2	6.1 mg/L	13.2 mg/L	17.0
4	11.0 mg/L	24.2 mg/L	31.2
8	15.3 mg/L	39.5 mg/L	51
12	20.7 mg/L	55.2 mg/L	71.2
24	20.7 mg/L	75.9 mg/L	97.5
250 μm GSH			
0	0 mg/L	0 mg/L	0
0.5	2.1 mg/L	2.1 mg/L	2.7
1	2.4 mg/L	4.5 mg/L	5.8
2	4.9 mg/L	9.4 mg/L	12.1
4	7.1 mg/L	16.5 mg/L	21.2
8	9.2 mg/L	25.7 mg/L	33.1
12	8.5 mg/L	34.2 mg/L	44.1
24	14.1 mg/L	48.3 mg/L	62.3
5 μm GSH			
0	0 mg/L	0 mg/L	0
0.5	0.6 mg/L	0.6 mg/L	0.8
1	0.9 mg/L	1.5 mg/L	1.9
2	0.9 mg/L	2.4 mg/L	3.1
4	1.1 mg/L	3.5 mg/L	4.5
8	1.4 mg/L	4.9 mg/L	6.3
12	4.5 mg/L	9.4 mg/L	12.1
24	7.7 mg/L	17.1 mg/L	22.0
PBS			
0	0 mg/L	0 mg/L	0
0.5	0 mg/L	0 mg/L	0
1	0.3 mg/L	0.3 mg/L	0.4

2	0.4 mg/L	0.7 mg/L	0.9
4	0.9 mg/L	1.6 mg/L	2.1
8	1.1 mg/L	2.7 mg/L	3.5
12	1.3 mg/L	4 mg/L	5.1
24	3.8 mg/L	7.8 mg/L	10

Cell toxicity test

The cytotoxicity of **1**, cisplatin and **5** was evaluated by using colorimetric (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) MTT assays against Hela and 4T1 cells. Briefly, the cells were seeded into a 96-well microculture plate (Costar) at a density of 5×10^3 cells/well and incubated in 5% CO_2 at 37 ℃ overnight. Then **1**, cisplatin or **5** were added to the medium at various concentrations, and the cells were incubated in 5% CO_2 at 37 ℃ for 24, 48 and 72 hrs. At each designed time intervals, the medium were replaced by 200 μL fresh medium, followed by the addition of 20 μL of 5 mg/ml MTT solution. The absorbance of the resulting solution was read at 490 nm using a microplate reader (Thermo Fisher Scientific Inc.). And the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

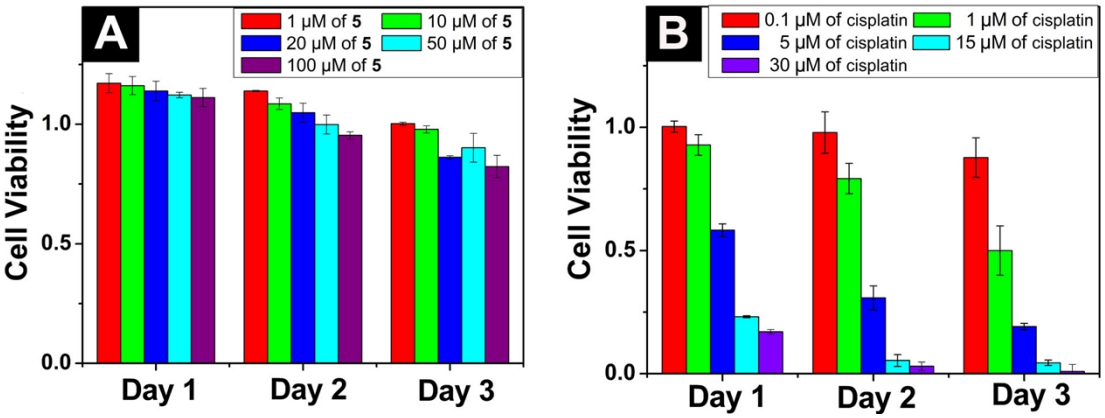


Figure S14. Cell viability of Hela cells treated with **5** (A) and cisplatin (B) for 3 days at different concentration.

Fluorescent confocal imaging

Hela cells at a density of 1×10^4 cells/well were seeded onto 8-mm diameter cell climbing slices placed in a 48-well microculture plate (Costar) and maintained in 5% CO_2 at 37 ℃ overnight prior to treating with **1**. After cells were incubated with **1** at a concentration of 30 μM for 24 h, CaspGlowTM Red Active Caspase-3 Staining Kit (BioVision, USA) was used according to the manufacturer's protocol to detect the apoptosis of Hela cells caused by **1**. A control experiment was prepared by treating the cells with both caspase-3 activation probe and caspase-3 inhibitor. Cell nuclei

were stained by 0.5 μg/mL DAPI (Invitrogen). The cellular imagining was performed by using confocal laser scanning microscope (Leica TCS SP5 II). Excitation/Emission wavelengths 405/440-480, 488/500-550 nm were for DAPI and caspase probe, respectively.

In vivo efficacy

All animal experiments were carried out in full compliance with the Animal Care and Use regulations of Soochow University. 4T1 cells (1×10^6 cells/each mouse) were injected subcutaneously into the flanks of female mice. When the tumors reached a size of 40~60 mm^3 (about 5 days after transplantation), 20 μL PBS, cisplatin (10 mg/kg) and **1** (40.2 mg/kg) were injected directly into the tumor on 0, 2, 4 and 6 days, respectively. The tumor volume (V) was calculated as follows: $V=L\times W^2/2$, where W is the tumor measurement at the longest point. The tumor volumes are normalized against the original volumes at 0 day for monitoring the tumor growth.

Biodistribution

4T1 cells (1×10^6 cells/each mouse) were subcutaneously transplanted into the flanks of female mice (16~18 g) for constructing the tumor-bearing mice. Then, the tumors were injected directly with PBS as a control, cisplatin (10 mg/kg) and **1** (40.2 mg/kg), respectively. Then, the various tissues including heart, liver, spleen, lung, kidney and tumor were extracted from the mice at 24 h post-injection, respectively. All the tissues were cut by high-speed homogenate machine and digested with nitric acid, and the diluted solution (0.4 mL of nitric acid and 3.6 mL H_2O) was measured by ICP-MS.

Histological analysis

The removed tumors and major organs were fixed immediately 4% formaldehyde solution for 24 h at room temperature. The tissues were frozen and sections (10 μM in thickness) cut on a cryostat. Hematoxylin and eosin (H&E) staining (BBC Biochemical, Mount Vernon, WA) was performed following manufacturer's instructions and the results were observed by an IX73 bright field microscopy (Olympus).

Reference:

1. E. A. Ottinger, L. L. Shekels, D. A. Bernlohr and G. Barany, *Biochemistry*, 1993, **32**, 4354.
2. S. Dhar, W. L. Daniel, D. A. Giljohann, C. A. Mirkin and S. J. Lippard, *J. Am. Chem. Soc.*, 2009, **131**, 14652–14653.
3. B. Ahn, J. Park, K. Singha, H. Park and W. J. Kim, *J. Mater. Chem. B*, 2013, **1**, 2829-2836.