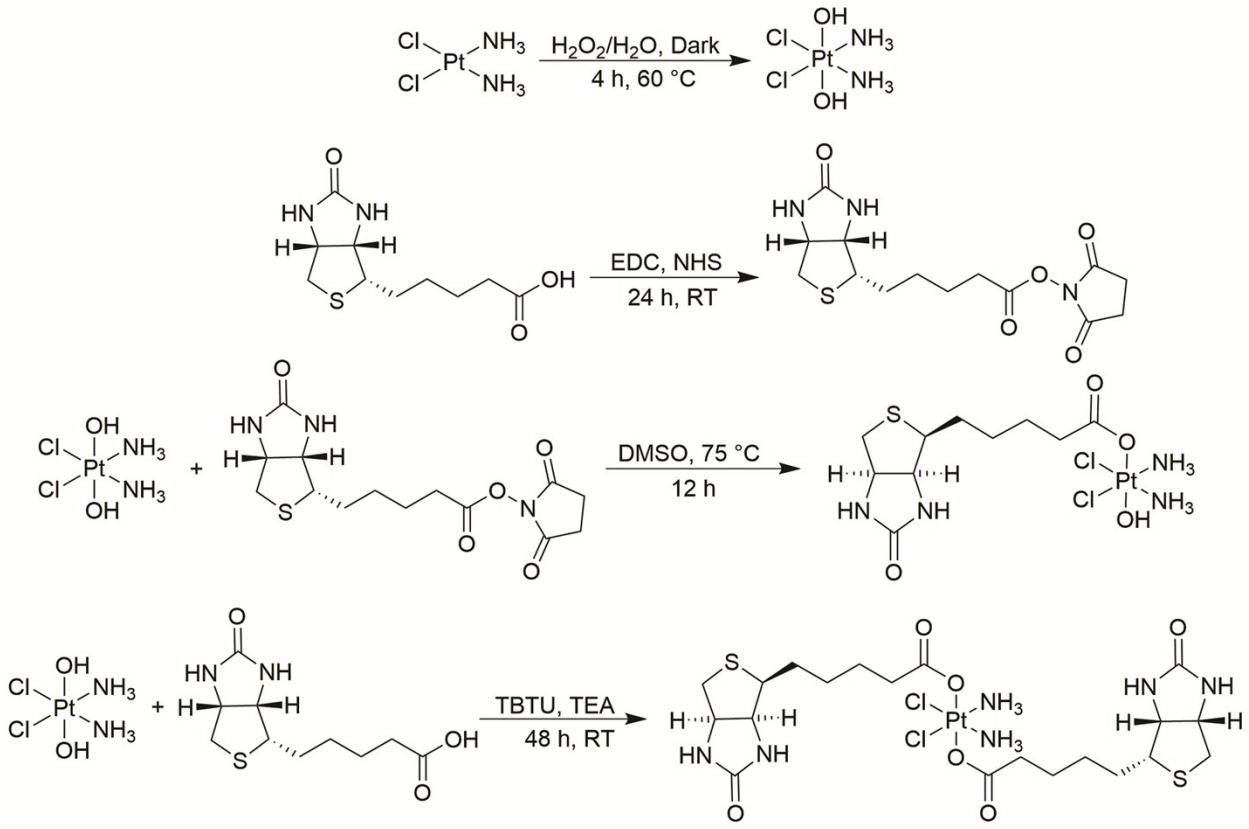
Electronic Supplementary Information (ESI)

Biotin-tagged platinum(IV) complexes as targeted cytostatic agents against breast cancer cells

Nafees Muhammad, Nasreen Sadia, Chengcheng Zhu, Cheng Luo, Zijian Guo* and Xiaoyong Wang*

1. Supplementary figures and tables



Scheme S1. Synthetic routes to Pt-Bio-I and Pt-Bio-II.

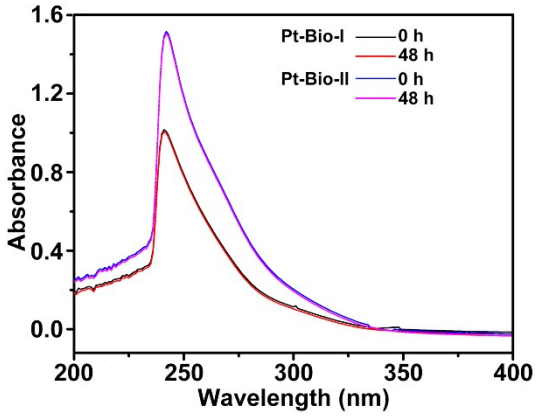
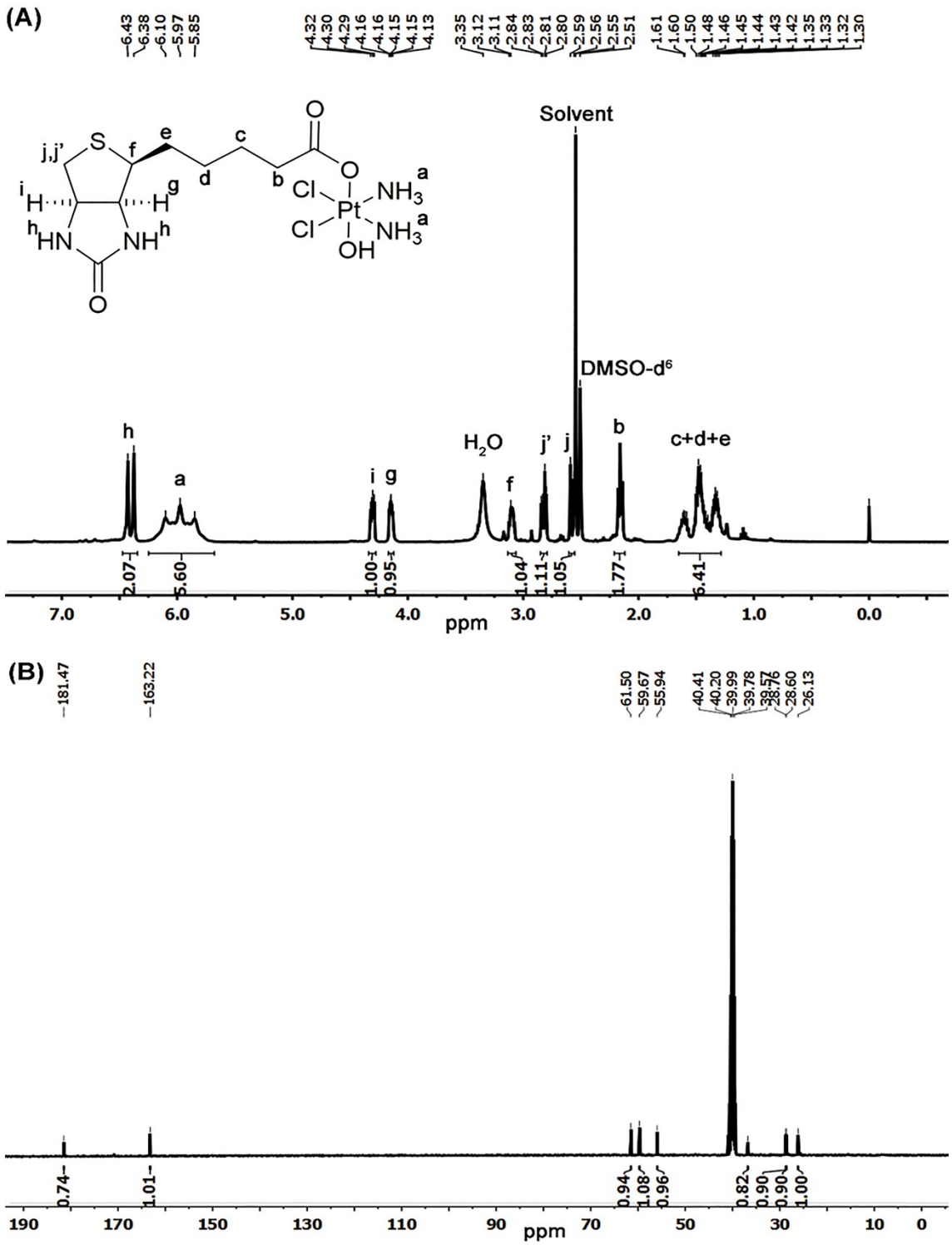


Fig. S1 UV-Vis spectra of Pt-Bio-I and Pt-Bio-II in water with 5% DMSO under physiological conditions (pH 7.4, 37°C) at 0 and 48 h.



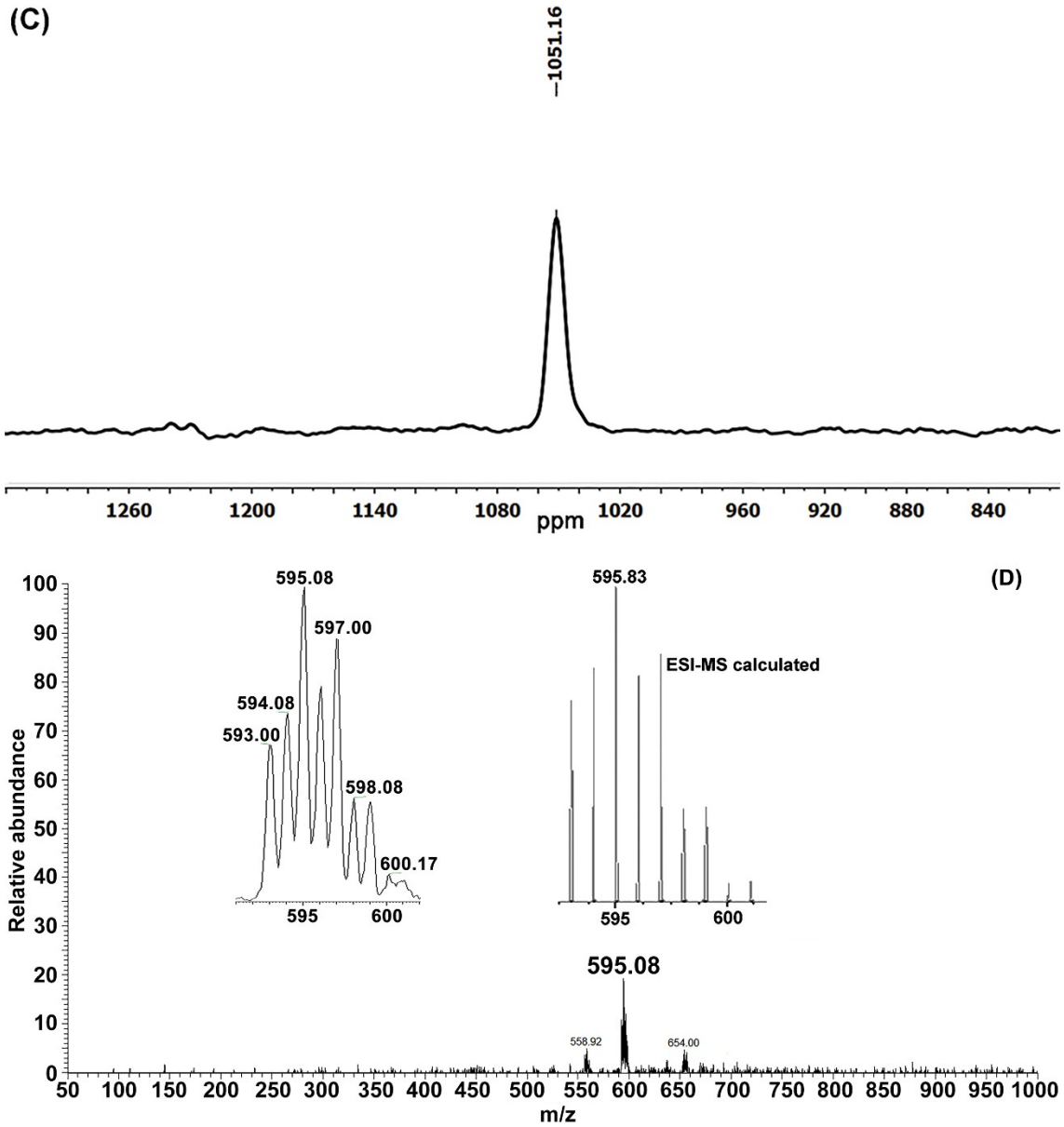
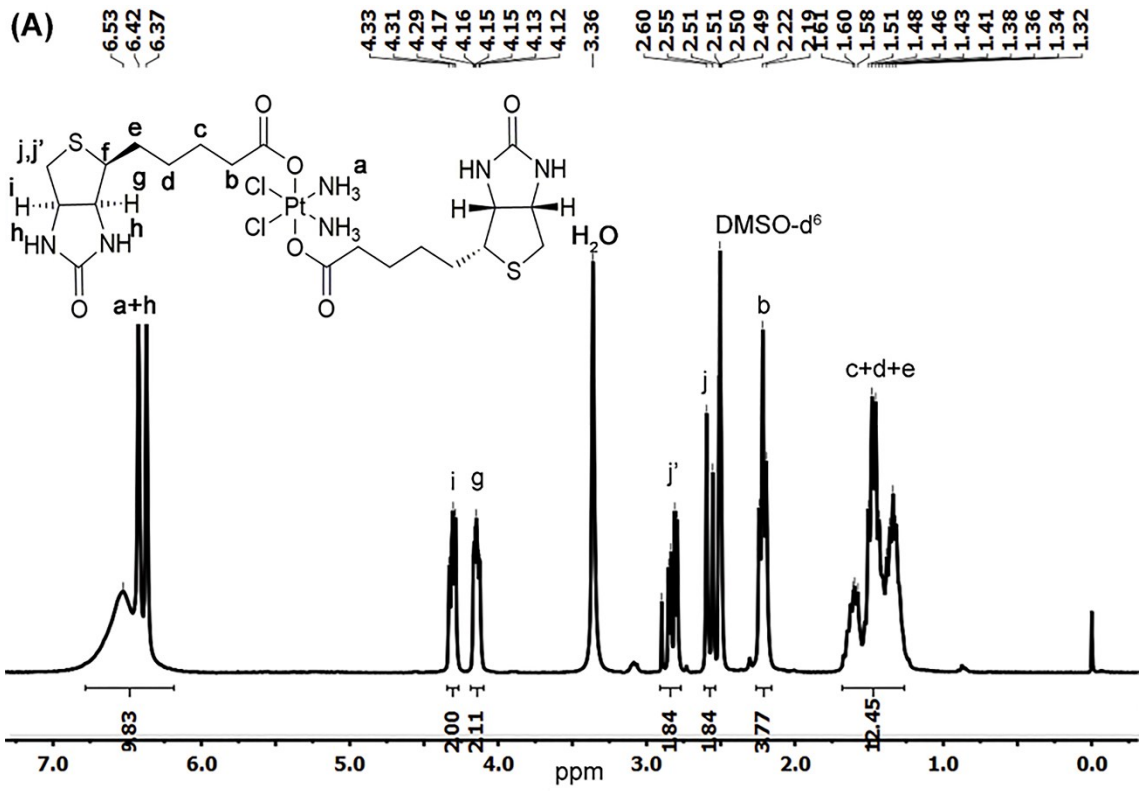
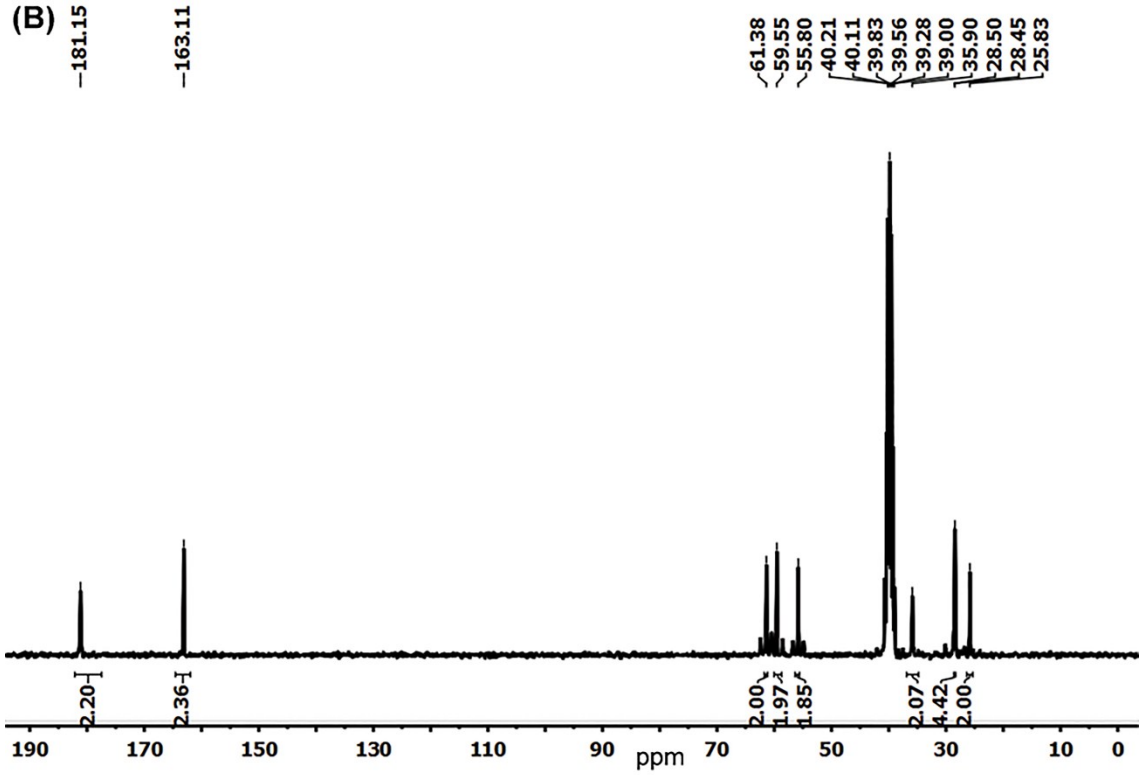


Fig. S2 ^{1}H-NMR (400 MHz, DMSO-d^{6}) (A), ^{13}C-NMR (400 MHz, DMSO-d^{6}) (B), ^{195}Pt-NMR (400 MHz, DMSO-d^{6}) (C) and ESI-MS (negative mode) spectra of Pt-Bio-I (D).


(A)
6.53
6.42
6.37
4.33
4.31
4.29
4.17
4.16
4.15
4.15
4.13
4.12
3.36
2.60
2.55
2.51
2.51
2.50
2.49
2.22
2.19
1.61
1.60
1.58
1.51
1.48
1.46
1.43
1.41
1.38
1.36
1.34
1.32
a+h
i
g
H2O
j'
j
DMSO-d6
b
c+d+e
9.83
2.00
2.11
1.84
1.84
3.77
12.45
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm


(B)
181.15
163.11
61.38
59.55
55.80
40.21
40.11
39.83
39.56
39.28
39.00
35.90
28.50
28.45
25.83
2.20
2.36
2.00
1.97
1.85
2.07
4.42
2.00
190
170
150
130
110
90
70
50
30
10
0
ppm

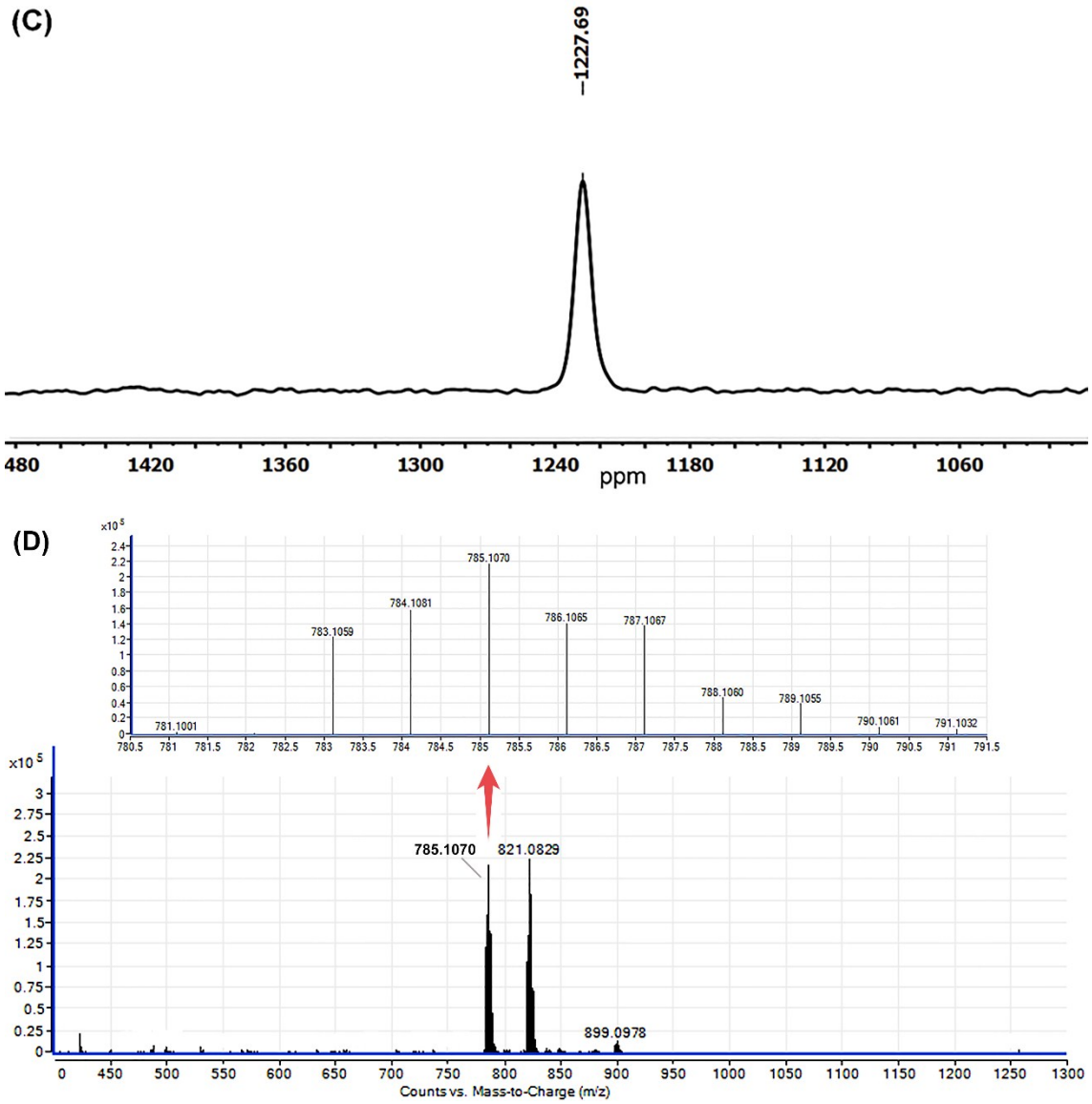


Fig. S3 ^{1}H-NMR (400 MHz, DMSO-d^{6}) (A), ^{13}C-NMR (400 MHz, DMSO-d^{6}) (B), ^{195}Pt-NMR (400 MHz, DMSO-d^{6}) (C) and HR-MS (negative mode) spectra of Pt-Bio-II (D).

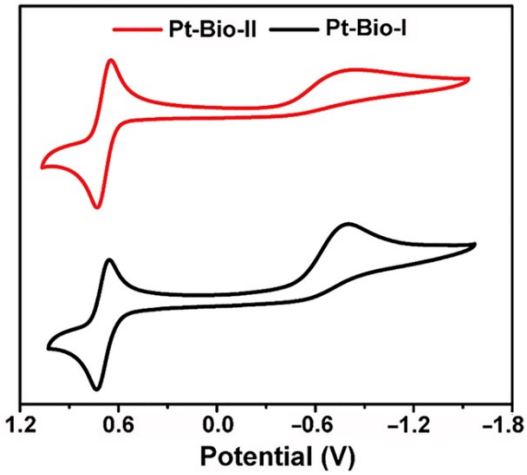


Fig. S4 Cyclic voltammogram of Pt-Bio-I and Pt-Bio-II in DMF containing 0.15 M (n-Bu_4N)PF_6 at a scan rate of 100 mV S^{-1} using a glassy carbon as a working electrode.

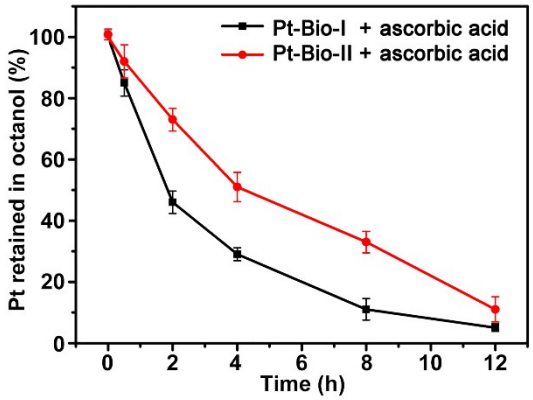


Fig. S5 Percentage of lipophilic Pt species in octanol after reduction of Pt-Bio-I and Pt-Bio-II (4 μM) by ascorbic acid (40 μM) at 37 ℃ for different periods of time in PBS.

Table S1. Pt distribution in MCF-7, MDA-MB-231 and MCF-10A/Vector cells (ng/10^6 cells) after incubation for 24 h.

Complex	MCF-7				MDA-MB-231				MCF-10A/Vector
	Total	*Nuclei*	*Cytoplasm*	*Membrane*	*Total*	*Nuclei*	*Cytoplasm*	*Membrane*	*Total*
Pt-Bio-I	27.5 ± 2.4	4.6 ± 1.7	13.6 ± 1.5	2.5 ± 0.6	25.4 ± 1.2	4.1 ± 1.1	8.7 ± 2.1	3.4 ± 1.4	5 ± 2
Pt-Bio-II	34.5 ± 0.9	1.6 ± 0.4	11.8 ± 2.1	8.7 ± 2.9	31.7 ± 0.8	2.3 ± 0.8	5.8 ± 0.5	12.7 ± 1.3	6.5 ± 2.9
Cisplatin	18.8 ± 2.3	2.9 ± 0.8	7.5 ± 1.7	3.7 ± 1.4	11.7 ± 1.9	1.5 ± 0.4	4.3 ± 1.7	2.3 ± 0.7	12 ± 1.8

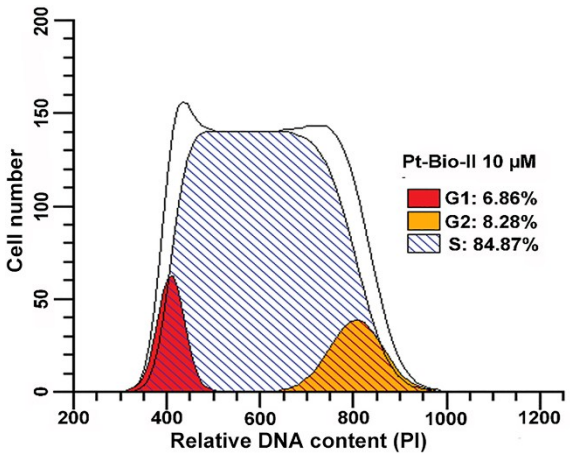


Fig. S6 Cell cycle arrest of MCF-7 cells treated with Pt-Bio-II for 24 h.

2. Experimental

2.1. Chemical reagents

All the reagents and solvents were of analytical grade and used as received without further purification. Cisplatin was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. China. Biotin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich and used as received without further purification. Other reagents were supplied by J&K Scientific, China and used as received. Water was deionized and ultrafiltered by a Milli-Q apparatus (Millipore Corporation, China). *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU), triethylamine (TEA), hydrogen peroxide (H_2O_2, 30%) was purchased from J&K Scientific. Annexin V conjugated with fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). Ascorbic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. Calf thymus (CT) DNA and DNAzol reagent was purchased from ThermoFisher Scientific.

2.2. Cell Lines

Human breast cancer MCF-7, human breast cancer MDA-MB-231, human mammary epithelial MCF-10A/vector, human liver cancer SMCC-7721 and human lung cancer A549 cell lines were purchased from American type culture collection (ATCC).

2.3. Instruments

Electrospray ionization mass spectra (ESI-MS) were obtained using an LCQ spectrometer (Finnigan). The isotopic distribution patterns for the complex were simulated using the ISOPRO 3.0 program. High resolution mass spectra (HR-MS) were measured on a liquid chromatography mass spectrometry instrument-G6500 (Agilent). ^{1}H-, ^{13}C-, ^{195}Pt-NMR spectra were acquired on a Bruker DRX-400 spectrometer at 298 K. The contents of Pt were determined on an inductively coupled plasma mass spectrometer (ICP-MS) using a standard Plasma-Quad II instrument (VG Elemental, Thermo OptekCorp.). Flow cytometry was determined by using Cytomics FC500 Flow Cytometry (Beckman Coulter Ltd.). CD spectra were recorded on a JASCO J-810 spectropolarimeter at room temperature using a cylindrical quartz cell (1.0 cm), with a continuous flow of nitrogen purging the polarimeter.

2.4. Synthesis of Biotin-NHS ester

Biotin-NHS was prepared by adding 1-ethy-l-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl) (584 mg, 3.05 mmol) and *N*-hydroxysuccinimide (NHS) (258 mg, 2.25 mmol) to a solution of D-Biotin (500 mg, 2.046 mmol) in anhydrous DMF (15 mL). The reaction mixture was stirred for 24 h at room temperature and then added into an ice-water mixture (100 mL). Biotin-NHS ester was precipitated out, which was washed with water (2x), methanol (2x) and then dried in vacuum. The final yield is 62%. ^{1}H-NMR in DMSO-d6, δ (ppm): 4.1 and 4.3 (m, 2H), 3.1 (m, 1H), 2.8 (dd, 5H), 2.6 (t, 2H), 2.59 (d, 1H) 1.3-1.7 (m, 6H) (see Fig. S7).

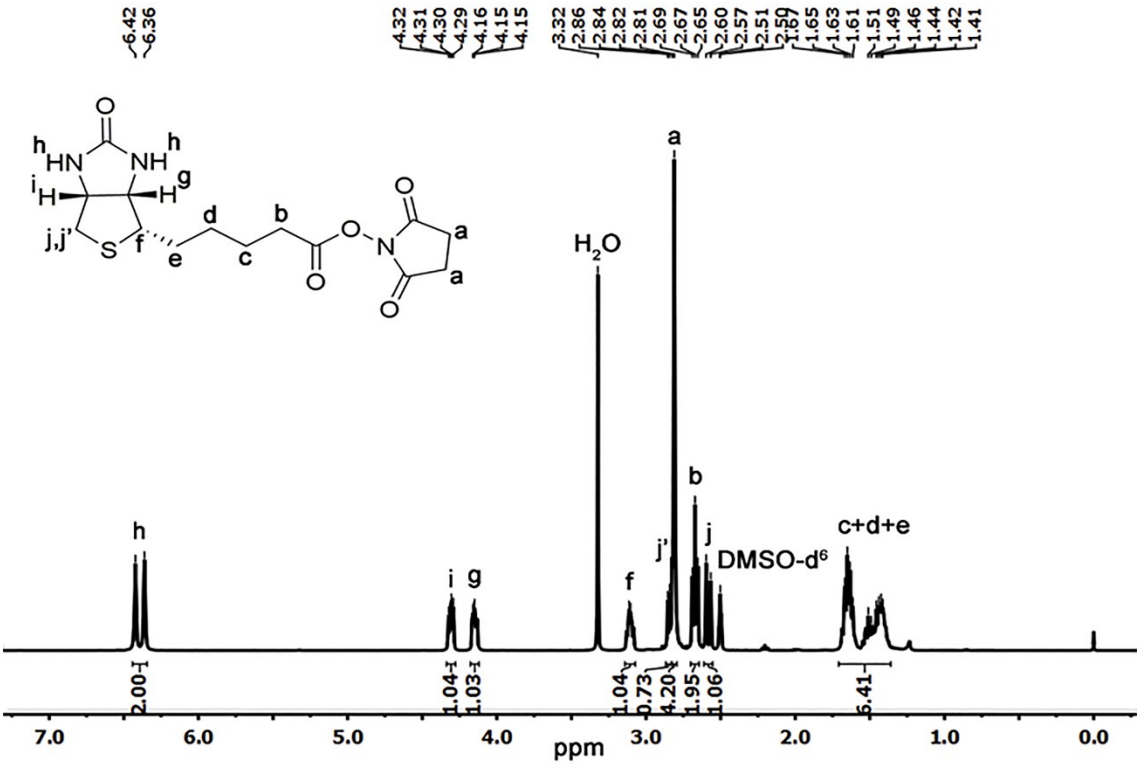


Fig. S7 ^{1}H-NMR (400 MHz, DMSO-d^{6}) spectrum of biotin-NHS ester.

2.5. Synthesis of Pt-Bio-I

Oxoplatin was synthesized by the oxidation of cisplatin with 30% H_2O_2 in a manner reported previously.[1] Yield: 64.90% (0.290 g, 0.868 mmol). IR 3460 (s, OH stretch), 1071 (m, Pt-OH bend), 556 (m, Pt-N(O) stretch). Biotin-NHS ester (180 mg, 0.52 mmol) was added to a suspension of oxoplatin (152 mg, 0.45 mmol) in DMSO (10 mL) and the reaction mixture was stirred overnight at 60 °C to form a clear yellow solution. DMSO was then removed by excessive addition of diethyl ether (100 mL). The final product was extracted with methanol and washed twice with methanol and ether and dried in vacuum. A light yellow solid product was obtained with a yield of 140 mg. ^{1}H-NMR (DMSO-d_6): δ (ppm) 1.3-1.7 (m, 6H), 2.15 (t, 2H), 2.59 (d, 1H), 2.7-2.8 (dd, 1H), 3.1 (m, 1H), 4.1 (m, 1H), 4.3 (m, 1H), 5.97 (m, 6H), 6.3-6.4 (d, 2H). ^{13}C-NMR (DMSO-d_6): δ (ppm) 26.13, 28.60, 28.76, 36.75, 55.94, 59.67, 61.50, 163.34, 181.54 ^{195}Pt-NMR (DMSO-d_6): 1051 ppm. ESI-MS (negative mode, m/z): $[M+Cl]^-$ = 595.08, calcd 595.83 $\{[Pt(NH_3)_2Cl_2(C_{10}H_{15}N_2O_3S)OH+Cl]^-\}$.

2.6. Synthesis of Pt-Bio-II

TBTU (360 mg, 1.12 mmol) and TEA (1 equivalent) were added to a solution of D-biotin (275 mg, 1.12 mmol) in DMF (15 mL). The reaction mixture was stirred for 30 min at room temperature followed by adding oxoplatin (150 mg, 0.449 mmol) and stirring for another 48 h. DMF was removed under high vacuum and the product was purified using column chromatography (silica, DCM/Methanol = 8:2, v/v). Pt-Bio-II was obtained as a white solid. Yield 95 mg. ^{1}H-NMR (DMSO-d^{6}): δ (ppm) 1.2-1.7 (m, 12H), 2.20 (t, 4H), 2.54 (d, 2H), 2.7-2.8 (dd, 2H), 3.1 (m, 2H), 4.1 (m, 2H), 4.2 (m, 2H), 6.3-6.8 (m, 10H). ^{13}C-NMR (DMSO-d^{6}): δ (ppm) 25.83, 28.63, 35.90, 55.80, 59.55, 61.57, 163.11, 181.15. ^{195}Pt-NMR (DMSO-d^{6}): 1227 ppm. HR-MS (negative mode, m/z): 785.1070, calcd 786.67 $\{[Pt(NH_3)_2Cl_2(C_{20}H_{30}N_4O_6S_2)]^-\}$.

2.7. Measurement of partition coefficient

Shake-flask method was used to measure the partition coefficient for complexes Pt-Bio-I and Pt-Bio-II. Briefly, a mixture of an equal volume of octanol and water was shaken on a mechanical shaker at room temperature for 24 h. Saturated solutions of both complexes in water (presaturated with n-octanol) were prepared in an ultrasonic bath followed by a filtration through a 0.2 μm nylon filter. About half of the stock solution (V_{water}) was mixed with a volume of water saturated octanol ($V_{octanol}$) and the mixtures were shaken for 2 h at room temperature. Centrifugation was carried out at 5000 g for 20 min to separate the phases. The aqueous layer was carefully separated and the Pt content was analysed in initial and final aqueous phases by ICP-MS. The log $P_{O/W}$ was measured using the following equation.

$$\log P_{O/W} = log\ (([C]initial - [C]final)/[C]final$$

2.8. Electrochemistry

A cyclic voltammogram was measured in a three-electrode cell using a glassy carbon disc working electrode, a platinum auxiliary electrode, and a platinum wire as a quasi-reference electrode, the potential of which was corrected using a trace amount of ferrocene/ferrocenium as internal reference standard. CHI660C electrochemical workstation was used for the measurement at room temperature. Deaeration of Pt-Bio-I and Pt-Bio-II solutions (2 mM in DMF) was accomplished by passing a stream of nitrogen through the solution for 10 min. [n-Bu_4N][PF_6] (0.15 M) was use as the supporting electrolyte. The potential was measured at a scan rate of 100mV s^{-1} and the reduction potentials were calculated vs. normal hydrogen electrode (NHE).

2.9. Reduction with ascorbic acid

Pt-Bio-I and Pt-Bio-II (4 μM) was added to a 2 mL PBS solution containing ascorbic acid (40 μM) and the resulting mixture was shaking on a shaker at 37 °C (600 rpm). After different time intervals (0, 0.5, 2, 4, 8, and 12 h), 200 μL of the sample was drawn out from these tubes and added to a new microcentrifuge tube containing 0.6 mL octanol. The mixture was vortexes for 10 min at R.T. and the two phases were separated by centrifugation at 8000 g for 2 min. The octanol extract was diluted with methanol for GFAAS measurement.

2.10. Cytotoxicity

The cytotoxicity was tested against three cancer cell lines: SMCC-7721 (liver carcinoma), A549 (lung carcinoma), MCF-7 (breast carcinoma) and MDA-MB-231 (breast carcinoma, cisplatin-resistant), while the human mammary epithelial cell line (MCF7-10A/vector) was used as a normal cell control. The SMCC-7721 and A549 cells were grown in 75 cm^2 cultural flask using RPMI 1640 growth medium supplemented with 10% fetal bovine serum. The MCF-7 and MCF-10A/vector cells were grown in 75 cm^2 cultural flask using DMEM/F-12 (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum. The MDA-MB-231 cells were grown in 75 cm^2 cultural flask using Leibovitz's L-15 growth medium supplemented with 14% fetal bovine serum. The cell cultures were incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The cytotoxicity of Pt-Bio-I, Pt-Bio-II and cisplatin were assessed using MTT [3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, cells were seeded in a 96 well plate at a density of 2000 cells/well, in 100 μL of growth medium and were preincubated for 24 h before exposure to the drugs. A stock solution of cisplatin were prepared in PBS while Pt-Bio-I and Pt-Bio-II stock solutions were prepared in DMSO. The stock solutions were diluted in a complete medium and then added in aliquots of 100 μL per well (DMSO concentration < 0.5%). After continuing exposure for 48 or 72 h, the cells were treated with MTT (20 μL, 5 mg mL^{-1} in PBS) for 4 h. The medium was removed and DMSO (200 μL) was added to dissolve the purple formazan crystals. The plates were shaken for 10 min and the absorbance of the solution was measured on a Varioskan flash multimode reader (Tokyo, Japan) at 570 nm. Each test was performed in triplicates.

Background information about MCF-10A/vector cells. A number of human mammary epithelial cell models have been developed to study breast cancer;[1,2,3] however, aggressive and metastatic human breast cell lines, such as MDA-MB-231 we tested in this study, do not have

matching normal cells. In this situation, we choose MCF-10A/vector cells tentatively as a control because it was suggested that the level of biotin receptors in cancer cell lines is greater than that in this cell line.[4] Some background information about MCF-10A/vector cells is given as follows.

The immortalized human mammary epithelial cell line MCF-10A is the most commonly used normal breast cell model. These cells were derived from benign proliferative breast tissue and spontaneously immortalized without defined factors. They are not tumorigenic and do not express estrogen receptor. They exhibit some features of normal breast epithelium, including lack of anchorage-independent growth and dependence on growth factors and hormones for proliferation and survival.[5]

MCF-10A/vector is the vector-transfected cell line that constitutively expressed empty vector. This cell line was generated by retroviral infection as described below. MCF-10A cells were plated at ~10^5 cells per well on a six-well plate. After 24 h, the medium was removed and 2 mL of the culture supernatant, which contained retroviral vectors and polybrene (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 8 μg mL^{-1}, was added to the wells. After 24 h of infection, the culture medium with viral supernatant was removed and replaced with fresh medium containing 2 μg mL^{-1} of puromycin for selection. MCF-10A cells that constitutively expressed empty vector were confirmed by western blot analysis.[6]

2.11. Flow cytometric analysis

Cell death was analysed by fluorescence-activated cell sorting (FACS) using Annexin V and propidium iodide (PI) staining assay. MCF-7 cells were seeded in a 6-well plate at a density of 2 × 10^5 cells per well and incubated in DMEM/F-12 incubation medium (2 mL) and allowed to settle for 24 h. The medium was replaced with the fresh one containing Pt-Bio-I, Pt-Bio-II and cisplatin respectively. After incubation for 48 and 72 h respectively, the cells were washed twice with cold PBS, trypsinized and centrifuged (4000 g, 3 min). The supernatant was discarded and the cells were resuspended in binding buffer (500 μL), stained with Annexin V and incubated in the dark for 15 min. The cells were treated with PI and analysed by flow cytometry.

2.12. Cell cycle

MCF-7 cells were seeded in a 6-well plate at a density of 2 × 10^5 cells per well and cultured in DMEM/F-12 medium and allowed to settle for 24 h. The medium was replaced with the fresh one containing cisplatin, Pt-Bio-I and Pt-Bio-II respectively. After incubation for 48 h, the cells were collected by trypsinization and washed with PBS, fixed in ice-cold ethanol (70%) for 12 h,

pelleted by centrifugation, and stained with PI in PBS for 30 min and then analyzed by flow cytometry using a FACS.

2.13. Cellular uptake

MCF-7, MDA-MB-231 and MCF7-10A/vector cells were seeded in a 6-well plate at a density of 2×10^5 cells/well. After 24 h incubation, the cells were treated with the compounds (10 μM) for 24 h. The attached cells were washed twice with PBS (4 °C). Cell pellets were collected by centrifugation and they were then digested with nitric acid (100 μL) for 2 h at 95 °C, followed by the addition of 50 μL of H_2O_2 and HCl (100 μL) to give a fully homogenized solution. Water was then added to dilute the solutions and the final Pt content was determined by ICP-MS. The Pt distribution in different cellular compartments (cytoplasm, nucleus and membrane) was determined using a FractionPREP cell fractionation kit from KeyGen Biotech China.

2.14. DNA platination

MCF-7 and MDA-MB-231 cells were seeded in a 150 cm^2 cultural flask at a density of 2×10^7 cells/flask. After incubation at 37 °C for 24 h, the cells were treated with the complex (10 μM) for 24 h. The attached cells were washed twice with PBS (4 °C), harvested by trypsinization (0.5 mL) and washed with 1 mL PBS. Cell pallets were lysed in DNAzol (1 mL genomic DNA isolation reagent, ThermoFisher Scientific) and the genomic DNA was extracted from lysate with 0.5 mL pure ethanol by incubating the sample for 1–3 min at room temperature. The isolated DNA was washed with 75% ethanol and redissolved in NaOH (1 mL, 8 mM). The DNA concentration was determined using nanodrop spectrophotometer and the Pt content was quantified by ICP-MS.

2.15. CD spectroscopy

The conformational variations in CT-DNA upon binding to Pt-Bio-I and Pt-Bio-II in the absence and presence of ascorbic acid was analysed by CD spectra. CT-DNA stock solution was prepared by dissolving it in a buffer solution (5 mM Tris-HCl, 50 mM NaCl, pH 7.4), which was stored in a refrigerator overnight at 4°C to reach homogenous phase and used within 4 days. The concentration of CT-DNA was determined by nanodrop spectrophotometer by taking 6600 M-1cm-1as its absorption coefficient at wavelength of about 260 nm. CD spectra were recorded in the range of 235–320 nm at a scan speed of 10 nm/min. Samples were prepared by taking fixed concentration of CT-DNA (60 μM) in the absence and presence of Pt-Bio-I and Pt-Bio-II (20 μM) and incubated at 37 °C for 48 h in the dark. To determine the impact of ascorbic acid on the reduction of Pt-Bio-I and Pt-Bio-II and their subsequent binding to CT-DNA, samples were

prepared by using a fixed concentration (60 μM) of CT-DNA, Pt-Bio-I/Pt-Bio-II (20 μM), and ascorbic acid (40 μM) and incubated for 48 h at 37°C.

3. Reference

1. R. J. Pauley, H. D. Soule, L. Tait, F. R. Miller, S. R. Wolman, P. J. Dawson and G. H. Heppner, The MCF10 family of spontaneously immortalized human breast epithelial cell lines: models of neoplastic progression. *Eur. J. Cancer Prev.*, 1993, 3 (Suppl. 2), 67–76,
2. P. Briand, K. V. Nielsen, M. W. Madsen and O. W. Petersen, Trisomy 7p and malignant transformation of human breast epithelial cells following epidermal growth factor withdrawal. *Cancer Res.*, 1996. 56, 2039–2044.
3. M. R. Stampfer, A. Bodnar, J. Garbe, M. Wong, A. Pan, B. Villeponteau and P. Yaswen, Gradual phenotypic conversion associated with immortalization of cultured human mammary epithelial cells. *Mol. Biol. Cell,* 1997, 8, 2391–2405.
4. J. Su, F. Chen, V. L. Cryns and P. B. Messersmith, Catechol polymers for pH-responsive, targeted drug delivery to cancer cells. *J. Am. Chem. Soc.* 2011, 133, 11850–11853.
5. 5. H. D. Soule, T. M. Maloney, S. R. Wolman, W. D. Peterson Jr., R. Brenz, C. M. McGrath, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res*. 1990; 50, 6075–6086.
6. 6. X. Fang, Y. Cai, J. Liu, Z. Wang, Q. Wu, Z. Zhang, C. J. Yang, L. Yuan and G. Ouyang, Twist2 contributes to breast cancer progression by promoting an epithelial–mesenchymal transition and cancer stem-like cell self-renewal. *Oncogene*, 2011, 30, 4707–4720.