Supporting information

NF-κB hijcking theranostic Pt(II) complex in cancer therapy

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Content

Materials and general instruments

All buffer components were of biological grade and were used as received. All chemical agents were commercially available and were used without further purification. Stock solutions of cisplatin (2 mM) were prepared in phosphate buffered solution (PBS) before dilution in cell medium.

Elemental analyses were performed with a perking Elmer240C elemental analyzer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer, and the chemical shifts are reported as parts per million from TMS (δ).Mass spectra were acquired on MALDI-TOF-MS (Bruker Autoflex III TOF/TOF*). UV-*vis* absorption spectra were recorded on a UV-265 spectrophotometer (concentration 1×10^{-5} mol/L). Photoluminescence measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer with a 450 W Xe lamp. Photoluminescence lifetime was studied on Horiba Fluoro Max-4P by using a LED lamp as the excitation source. The protein structures employed for the present studies were IKBBETA/NF-KB P65 HOMODIMER COMPLEX (LOY3) and NF-kappaB p65 subunit dimerization domain homodimer (1MY5). The methodology applied in this study was ligandfit by using Discovery Studio 4.1 software.

The X-ray diffraction measurements were performed on a Bruker SMART CCD area detector using graphite monochromated Mo-K_α radiation (λ = 0.710698 Å) at 298(2) K. Intensity data were collected in the variable ω-scan mode. The structures were solved by direct methods and difference Fourier syntheses. The non-hydrogen atoms were refined anisotropically and hydrogen atoms were introduced geometrically. Calculations were performed with a SHELXTL-97 program package.

Synthesis of 2,4,6-triphenylpyridine

10.6 g (0.1 mol) benzaldehyde, 24.0 g (0.2 mol) acetophenone, 10.6 g (0.2 mol) ammonium chloride and 200 mL acetic acid were added in a 500 mL three-neck flask. The mixture was heated to reflux for 24 h, and then cooled to room-temperature.

The crude product was achieved by filtration and re-crystalized from ethanol to give 26.1 g white powder. Yield: 85 %.

Synthesis of Pt complex

0.307 g (1 mmol) 2, 4, 6-triphenylpyridine and 0.498 g (1.2 mmol) K_2PtCl_4 were mixed in 150 mL acetic acid. The suspension liquid was heated to 90 °C for 72 h under nitrogen atmosphere. A yellow-green filter residue would be obtained after filtration. The crude intermediate, without further purification, was dissolved in 5 mL DMSO and refluxed for 30 min. After that, the reaction solution was treated with 50 mL deionized water. A yellow powder would be achieved after filtration and dried under vacuum condition. The purified product was obtained by column chromatography (neutral alumina, hexane:ethyl acetate = 5:1) as yellow powder (0.416 g). Yield: 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (dd, J = 7.5, 1.0 Hz, 2H), 7.74 – 7.69 (m, 2H), 7.60 – 7.47 (m, 7H), 7.31 (td, J = 7.4, 1.3 Hz, 2H), 7.15 (td, J = 7.5, 1.3 Hz, 2H), 3.80 – 3.60 (m, 6H). ^{13}C NMR (400 MHz, $CDCl_3$) 29.0, 118.0, 127.0, 127.4, 127.6, 129.0, 129.3, 138.0, 140.0 152.0, 158.MALDI-TOF-MS m/z: Calculated for M/z = 578.10. Found m/z = 578.10. Anal. Calcd. for $C_{25}H_{21}NOPtS$: C, 51.90; H, 3.66; N, 2.42. Found: C, 51.86; H, 3.64; N, 2.44.

Cytotoxicity assay

To determine the cytotoxic effect of Pt complex and cisplatin which treated over 48 h as a period, the 5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay was performed. The Pt complex was dissolved in DMSO, while cisplatin was dissolved in PBS. When HepG2/HELF cells reached ~70 % confluence, HepG2/HELF cells were harvested by trypsin and plated in flat-bottom 96-well plates for 24 h. Prior to the treatment of Pt complex and cisplatin, the medium was removed and replaced with fresh medium/stock solution = 99/1 (containing concentration of Pt complex and cisplatin 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 μM). Subsequently, the treated cells were incubated for 48 h at 37 °C with 5% CO_2. After

that, the cells were treated with 5 mg/mL MTT (40μL/well) and incubated for another 4 h (37 °C, 5% CO_2). Then medium was removed, the formazan crystals were dissolved in DMSO (150 μL/well), and the absorbance at 490 nm was recorded. The cell viability (%) was calculated according to the following equation: cell viability % = OD_{490} (sample)/OD_{490} (control) ×100, where OD_{490} (sample) represented the optical density of the wells treated with various concentrations of the compounds and OD_{490} (control) represented that of the wells treated with DMEM + 10% FCS. Each concentration of Pt complex and cisplatin covered eight wells which was considered as one experimental group. And the averages and standard deviations were also reported. The reported percent of cell survival values are related to untreated control cells.

Low temperature and inhibitors studies

HepG2 cell was cultured in glass-bottom dish for 48 h, after that the cell was washed by PBS for 3 times and then cultured at 4 °C for 30 min. After that, 5 μL Pt complex (1×10^{-3} M in DMSO) was added to the plate and cultured for another 1 h. For inhibitors study, cells incubated with 1 μM of inhibitors (including 2-deoxy-D-glucose, NH_4Cl, chloroquine, nocodazole, chlorpromazine, and colchicine, which dissolved in 1 mL culture medium without serum) for 30 min (37 °C, 5 % CO_2), then 5 μL Pt complex (1×10^{-3} M in DMSO) was added to the plate and cultured for another 1 h (37 °C, 5 % CO_2). The imaging was carried out after the cells were washed by PBS for 3 times.

Confocal cell imaging

Confocal microscopy imaging was acquired with a Carl Zeiss LSM 710 confocal microscopy and 63X/100X oil-immersion objective lens. The incubated cells were excited at 405 nm for Pt complex, 550 nm for Cy3, 405 nm for DAPI, 535 for PI, 488 for FITC, 633 nm for Cy5 with a semiconductor laser and the emission signals were collected at 480-520 nm for Pt complex, 560-600 nm for Cy3, 420-460 nm for DAPI,

600-640 nm for PI, 500-540 nm for FITC, 640-680 nm for Cy5, respectively. Quantization by line plots was accomplished by using the software package provided by Carl Zeiss instrument.

Immunofluorescence

The cell (RAW264.7, HepG2, and Astrocyte) were cultured in the glass-bottom dish for 48 h, after that the cell were treated with 10 μL Pt complex (1×10^{-3}mol/L, in DMSO) for 1 h. The cell were washed by PBS for 3 times and fixed with 4% formaldehyde in PBS for 15 min at room temperature. The following protocol was block specimen in Blocking Buffer (1 × PBS/5 % normal serum/0.3 % Triton^{TM}X-100 for 60 min). Washed in PBS 3 times, 5 min each time. Cells were then incubated with the primary antibody (Anti-NF-κB p65: ab16502, Anti-GFAP antibody: ab68428) diluted in Antibody Dilution Buffer at 4℃ overnight. Washed in PBS 3 times, 5 min each time. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for 1-2 hrs at room temperature in the dark. Wash by PBS for 3 times, and then coverslip slides with mounting medium. For long-term storage keep slides flat at 4 °C protected from the light.

Transmission Electron Microscope (TEM) cell imaging

For TEM, HepG2 cells and HELF cells were incubated with Pt complex then fixed by using 3% glutaraldehyde and dehydrated with ethanol. For control cells, secondary fixation was carried out in 1 % aqueous osmium tetroxide for 1 hour at room temperature, in order to visualize the membrane structures. The detailed protocols were listed as follow: For transmission electron microscopy, Cell specimens were received pelleted in Eppendorf tubes. Fresh 3 % glutaradehyde in 0.1 M phosphate buffer was added to re-suspend the pellet to ensure optimal fixation, and left overnight at 4 ^{o}C. The specimens were then washed in 0.1 M-phosphate buffer at 4 ^{o}C, twice at 30 min intervals. Secondary fixation was carried out in 2 % aqueous osmium tetroxide for 2 hours at room temperature, followed by washing in buffer as

above. Continuing at room temperature, this was followed by dehydration through a graded series of ethanol: 75 % (15 min), 95 % (15 min), 100 % (15 min) and 100 % (15 min). 100 % ethanol was prepared by drying over anhydrous copper sulphate for 15min. The specimens were then placed in an intermediate solvent, propylene oxide, for two changes of 15min duration. Resin infiltration was accomplished by placing the specimens in a 50/50 mixture of propylene oxide/Araldite resin. The specimens were left in this mixture overnight at room temperature. The specimens were left in full strength Araldite resin for 6-8 hrs at room temperature (with change of resin after 3-4 hrs) after which they were embedded in fresh Araldite resin for 48-72 hrs at 60 °C. Semi-thin sections approximately 0.5 µm thick were cut on a Leica 10 ultramicrotome and stained with 1 % Toluidine blue in Borax. Ultra-thin sections, approx. 70-90nm thick, were cut on a Leica ultramicrotome and stained for 25min with saturated aqueous uranyl acetate followed by staining with Reynold's lead citrate for 5mins. The sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80kV. Electron micrographs were taken using a Gatan digital camera.

Flow cytometry

Before the experiment, the solutions of two reagents were prepared by dissolved the Pt complex and cisplatin in DMSO and PBS, respectively. Cells (including cancer cell and nomal cell) were cultured in 25 cm^2 culture flasks in DMEM, supplemented with fetal bovine serum (10%), penicillin (100 units/mL) and streptomycin (50 units/mL) at 37 °C in a CO_2 incubator (95% relative humidity, 5% CO_2). Cells were seeded in 35 mm glass bottom cell culture dishes, at a density of 1 × 10^5 cells and were allowed to grow when the cells reached more than 60% confluence. Different concentrations of Pt complex and cisplatin were added and the cells were incubated for 24 h. After that, the cells were trypsinized and washed with cold PBS twice. Apoptosis detection kit were used according to the specification. Then the samples were quantified by flow cytometry (Beckman Coulter, Inc.USA).

Animal method:

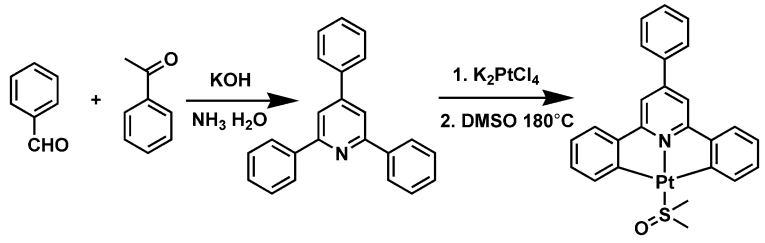
All procedures involving animals were approved by and conformed to the guidelines of the Southwest University Animal Care Committee, College of Pharmaceutical Sciences. We have taken great efforts to reduce the number of animal used in these studies and also taken effort to reduce animal suffering from pain and discomfort.

Cisplatin was purchased from Aladdin Company and dissolved in PBS before used. To develop murine breast cancer model, six to eight weeks old female BALB/c mice were subcutaneously injected at the right back with 0.1mL cell suspension containing 5×10^5 4T1 cells. When the tumor size was 50-100 mm^3, all the mice were randomly divided into six groups of six animals per group: (1) PBS (20 μL), (2) *Cisplatin* (3 mg/kg), (3) Pt complex (3 mg/kg). The injected doses were referred to previous work.[1, 2] The mice were treated *via* intratumoral injection. The mice of each group were treated with above agents at 1^{st}, 3^{rd}, and 5^{th} day. After administration, the tumor size was measured with caliper and body weight was recorded every the other day for 11 days. After that, all the mice were sacrificed. The major organs and tumors were excised and weighed. The tumor volume was calculated as the following equation: $V= (d^2\times l)/2$, where d and l were the width and length of the tumor, respectively. The tumor growth inhibition (TGI) was calculated using the equation of $TGI = W_0/W_t\times100\%$, where W_0 and W_t represented the tumor weight of PBS group and treated group, respectively.

Image processing and analysis

Micrographs were processing and analyzed by ZEISS Imaging Browser and ImageJ 1.48v (32-bit). Quantification of the fluorescence intensity was achieve via Analyze >> Tools >> ROI manager in ImageJ from three parallel experiments. Quantification of single cell intensity profile was achieve via Analyze >> Plot Profile by selecting one cell in ImageJ. Quantification of colocolizationcoefficency was

achieve *via* an external plugin *via* Plugins >>Colocolization Finder. For more details, please refer to online sources: https://imagej.nih.gov/ij/.



Scheme S1. The synthetic routes for platinum complex Pt complex.

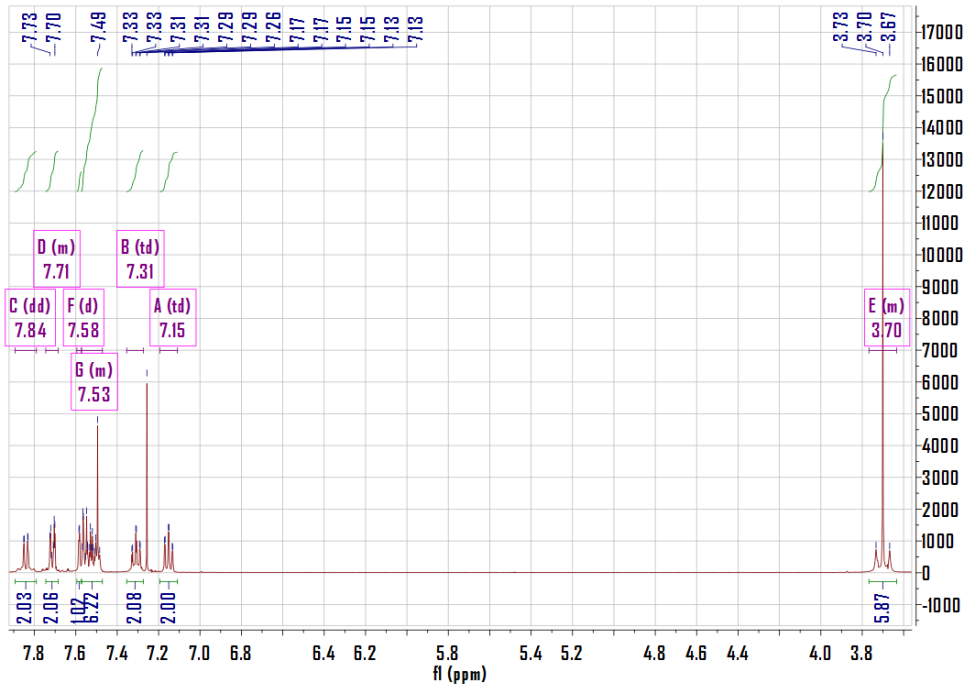


Figure S1. The ^{1}H NMR spectrum of Pt complex in *d*-$CDCl_3$.

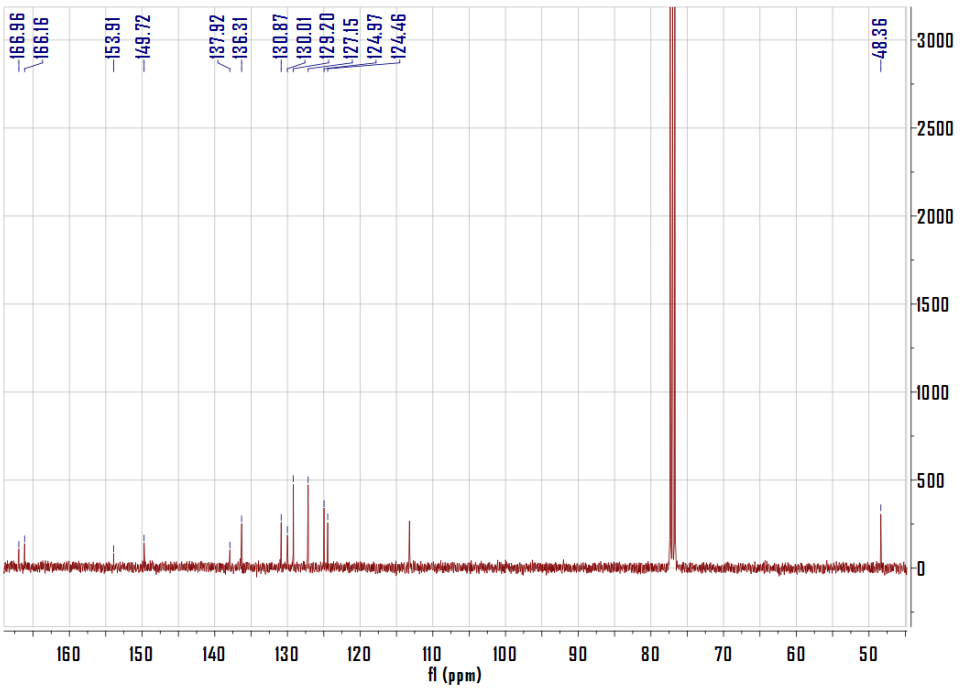


Figure S2. The ^{13}C NMR spectrum of Pt complex in $CDCl_3$.

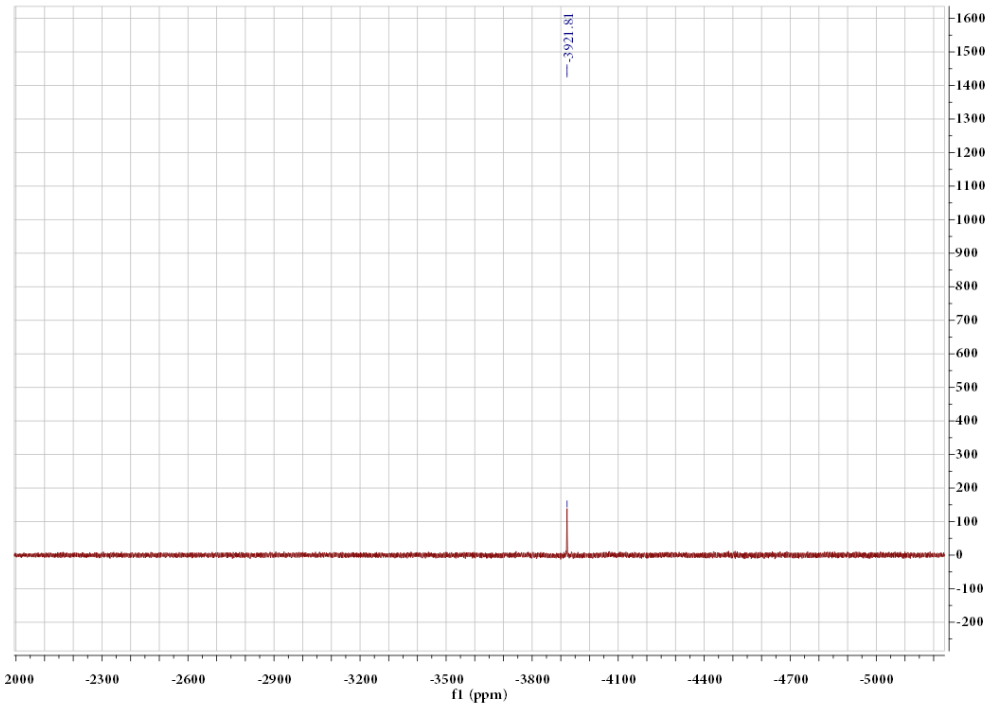


Figure S3. The ^{195}Pt NMR spectrum of Pt complex.

Table S1. Crystal data collection and structure refinement for Pt complex.

Complex	Pt complex
CCDC no.	1018679
Empirical formula	$C_{26}H_{22}Cl_3NOPtS$
Formula weight	697.95
Temperature	293(2) K
Wavelength	0.71073 A
Space group	Triclinic
Crystal system	$P\bar{1}$
a/Å	9.2597(9)
b/Å	9.9575(10)
c/Å	14.9798(14)
α(°)	70.9170(10)
β(°)	75.0880(10)
γ(°)	74.5370(10)
Volume A^3	1235.9(2)
Z	2
Dc/Mg m^{-3}	1.876
μ/mm^{-1}	6.106
F(000)	676
Final R indices[I > 2σ(I)]	R_1 = 0.0217, wR_2 = 0.0616
Goodness-of-fit on F2	1.068

Table S2. The selected bond length of Pt complex (Å)

Pt(1)-N(2)	2.007(3)	C(1)-C(6)	1.394(8)
Pt(1)-C(23)	2.069(4)	C(2)-C(3)	1.383(6)
Pt(1)-C(13)	2.102(4)	C(3)-C(4)	1.408(6)
Pt(1)-S(1)	2.187(1)	C(4)-C(5)	1.395(6)
S(1)-O(1)	1.487(3)	C(4)-C(7)	1.482(5)
S(1)-C(50)	1.776(4)	C(5)-C(6)	1.372(6)
S(1)-C(49)	1.779(5)	C(7)-C(8)	1.394(6)
N(2)-C(9)	1.352(5)	C(7)-C(10)	1.395(6)
N(2)-C(11)	1.353(5)	C(8)-C(9)	1.388(5)
C(1)-C(2)	1.365(7)	C(9)-C(18)	1.473(5)

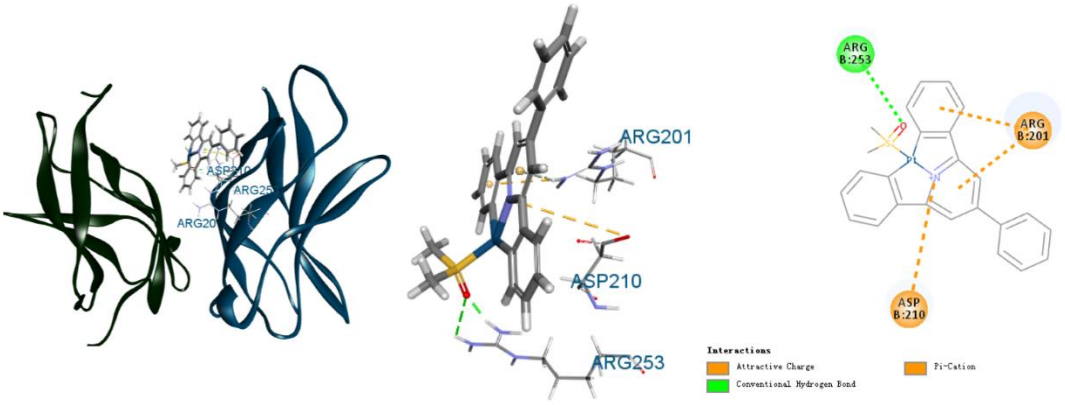


Figure S4. The intramolecular interaction between Pt complex and active NF-κB protein.

Table S3. The calculated docking data of Pt complex with inactive and active NF-κB protein

Protein Name	Binding Energy (kcal/mol)	Ligand Energy (kcal/mol)	Protein Energy (kcal/mol)	Complex Energy (kcal/mol)	Entropic Energy (kcal/mol)
Inactive protein	-37.53	60.08	23886584.53	23886607.08	20.61
Active protein	-30.53	60.48	-8660.46	-8630.52	20.59

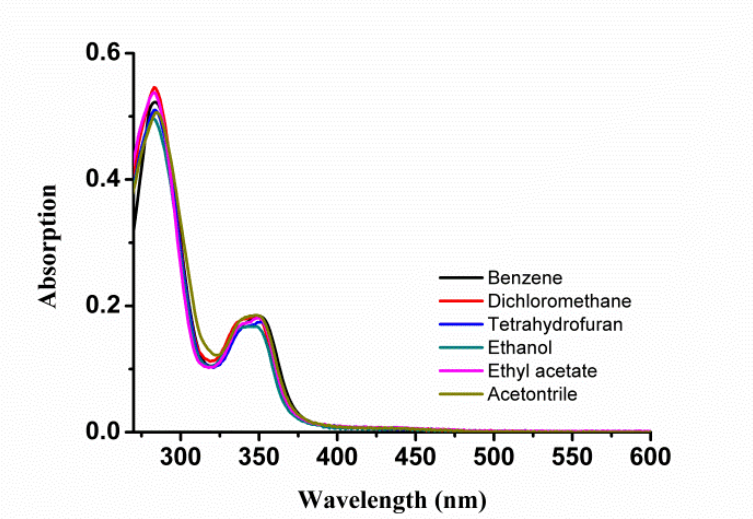


Figure S5. The absorption spectra of Pt complex in different solutions with concentration 1×10^{-5} M.

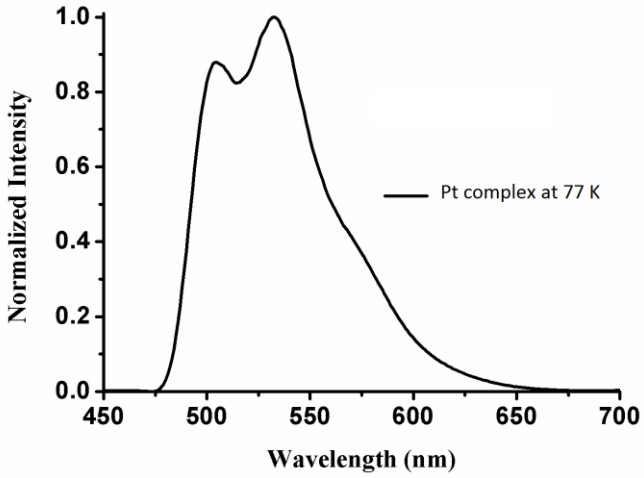


Figure S6. The normalized emission spectrum of Pt complex in 2-Methyltetrahydrtofuran at 77K.

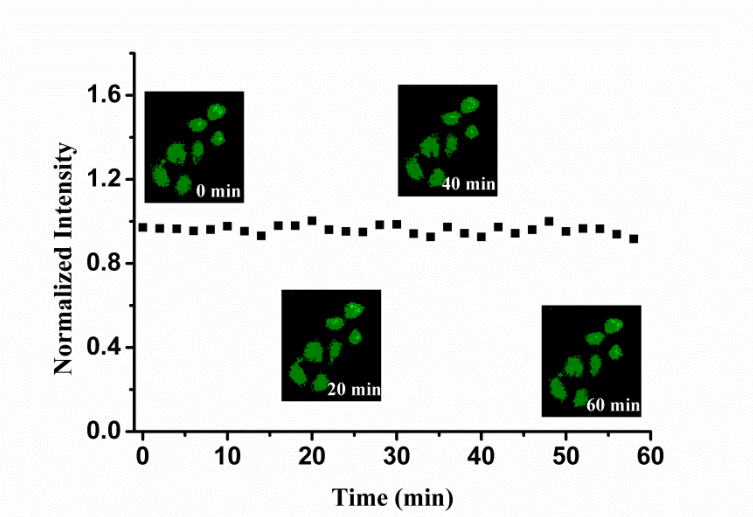


Figure S7. Time series fluorescence intensity of Pt complex in HepG2 cells (20μM, 30 min) under laser exposure over 60 min, inset imaging from times point 0, 20, 40, and 60 min (excited at 405 nm).

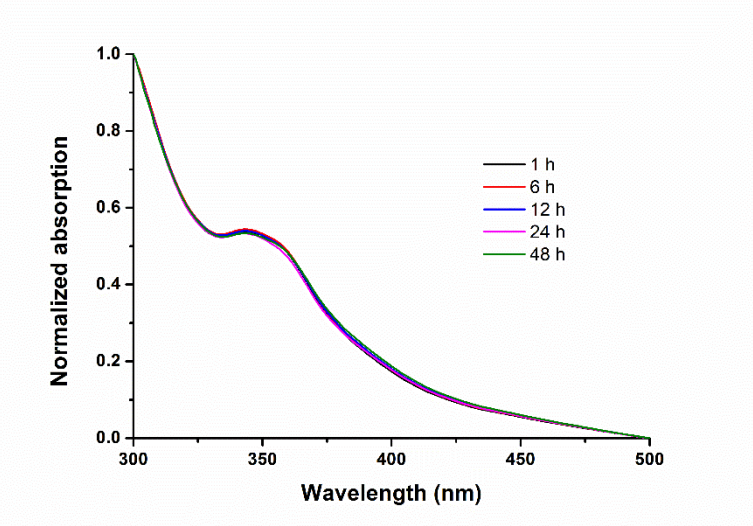


Figure S8. The UV-*vis* absorption spectra of Pt complex dissolved in cell culture media for different time (1, 6, 12, 24, 48 h).

Table S4. The ICP-MS quantification of internalized cisplatin in nucleus and cytosol of HELF and HepG2 cells.

Cell type	Organelle	Pt content (pg/cells)
HELF	Nucleus	19.46
	Cytosol	4.65
HepG2	Nucleus	22.37
	Cytosol	4.23

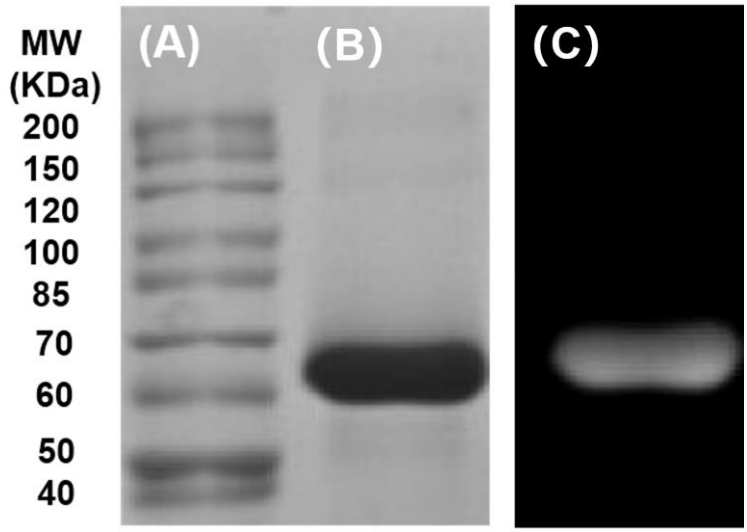


Figure S9. Protein electrophoresis analysis of NF-κB proteins upon staining with Coomassie brilliant blue complex G-250 and Pt complex. Lane A was the marker. Lane B was the NF-κB protein stained with Coomassie brilliant and Pt complex. The photograph was taken at bright field. Lane C was the NF-κB protein stained with Pt complex (10^{-4} M) at 37 °C for 2h (excited at 365 nm).

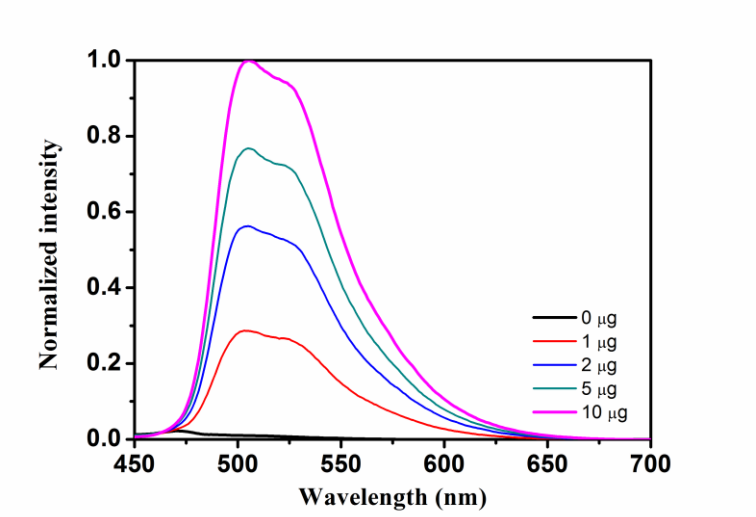


Figure S10. The normalized emission spectra of different quality NF-κB protein mixed with Pt complex (1×10^{-5} M) in PBS.

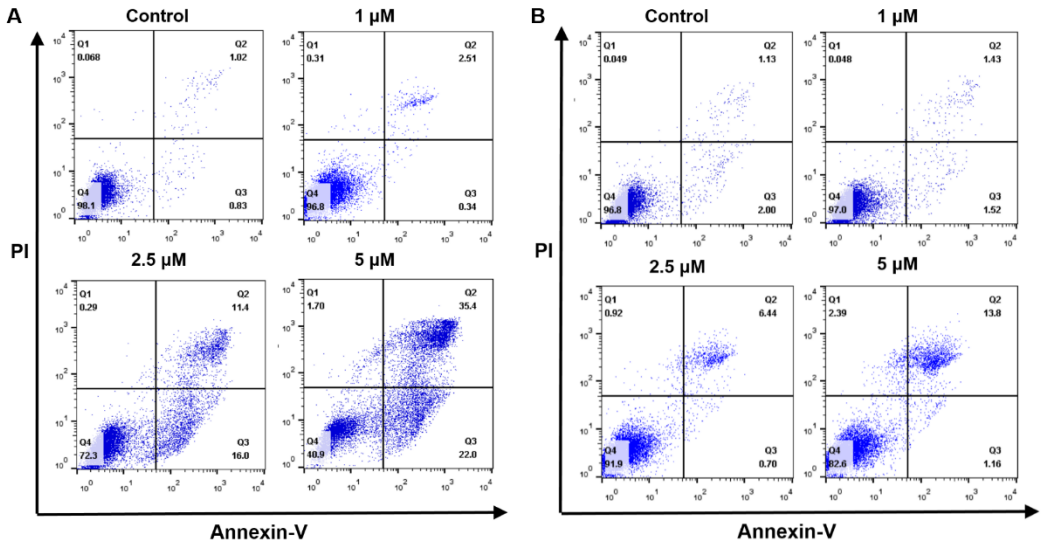


Figure S11. Flow cytometry analysis for apoptosis of Pt complex against HepG2 cancer cells (A) and HELF normal cells (B) at different concentration (0 μM, 1 μM, 2.5 μM, and 5μM) for 24 h. Q1, necrotic cells;Q2, late apoptotic cells; Q3, early apoptotic cells; Q4, living cells. Inserted numbers in the profiles indicate the percentage of the cells present in this area.

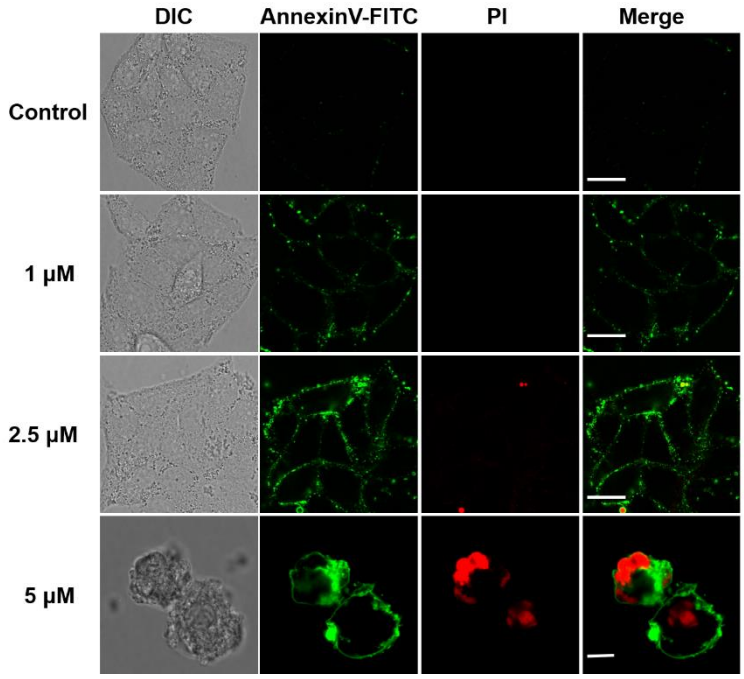


Figure S12. The cancer cells treated with Pt complex at different concentration (0 μM, 1 μM, 2.5 μM, and 5μM).

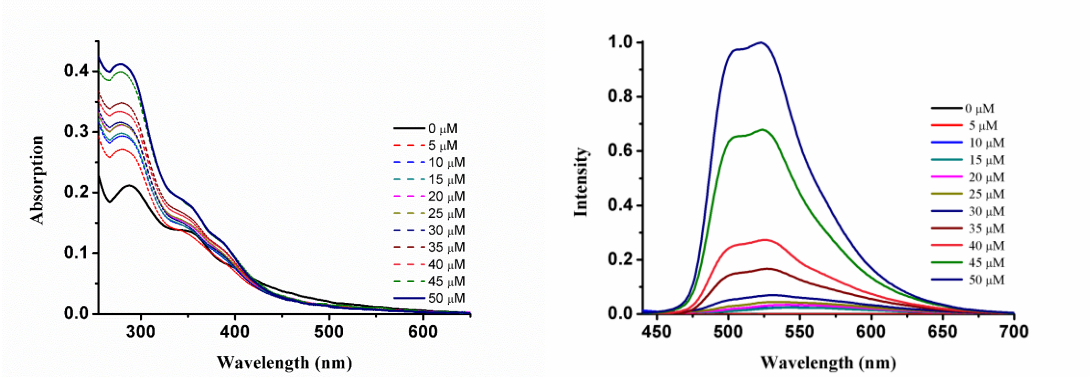


Figure S13. The UV-vis absorption spectra (left) and emission spectra (right) of Pt complex (1×10^{-5} M) titrated with different concentration of DNA (0-50 μM).

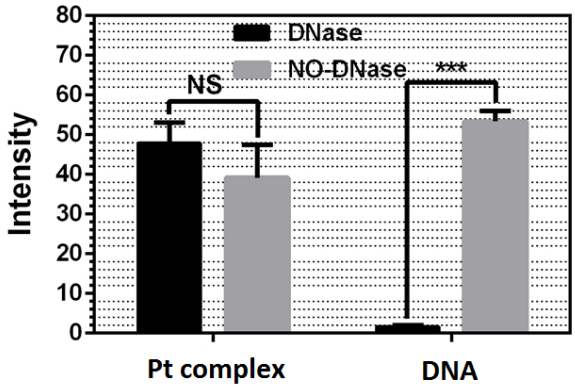


Figure S14. The emission intensity of Pt complex and commercial dyes treated with/without DNase.

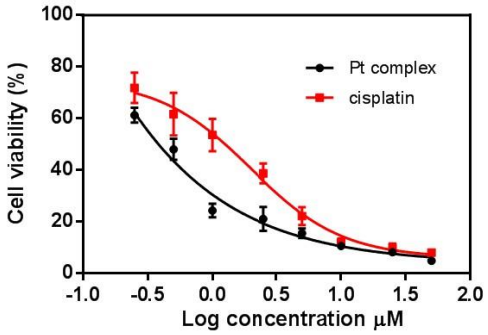


Figure S15. The Cytotoxicity of Pt complex (black line) and cisplatin (red line) (0.25 – 50 μM) against 4T1 cell lines (48 h incubation time).

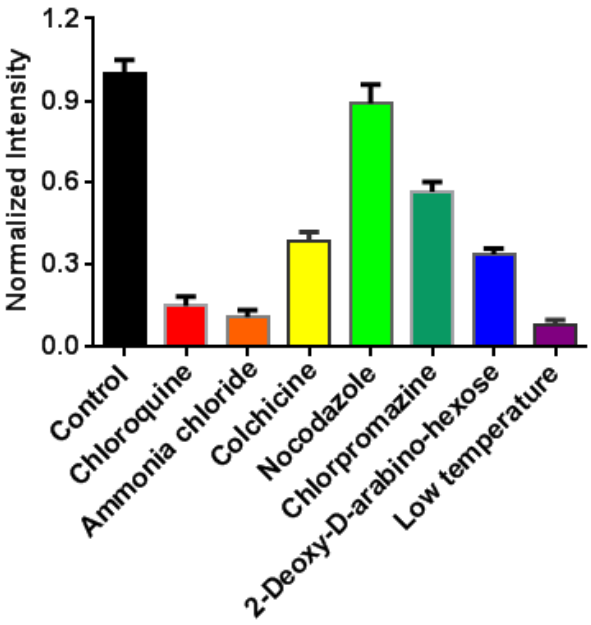


Figure S16. The cell uptake inhibited experiment of Pt complex with six typical inhibitors and low temperature.

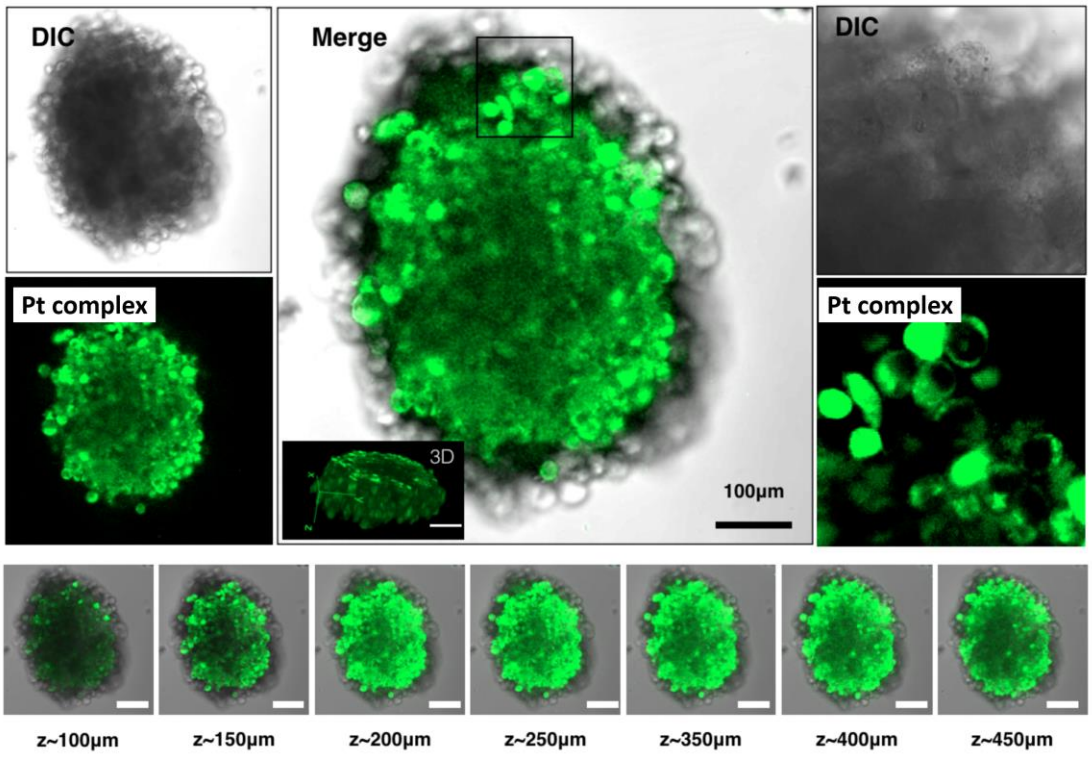


Figure S17. Confocal imaging of 3D HepG2 cancer cells multicellular tumor spheroids treated with Pt complex over 30min.

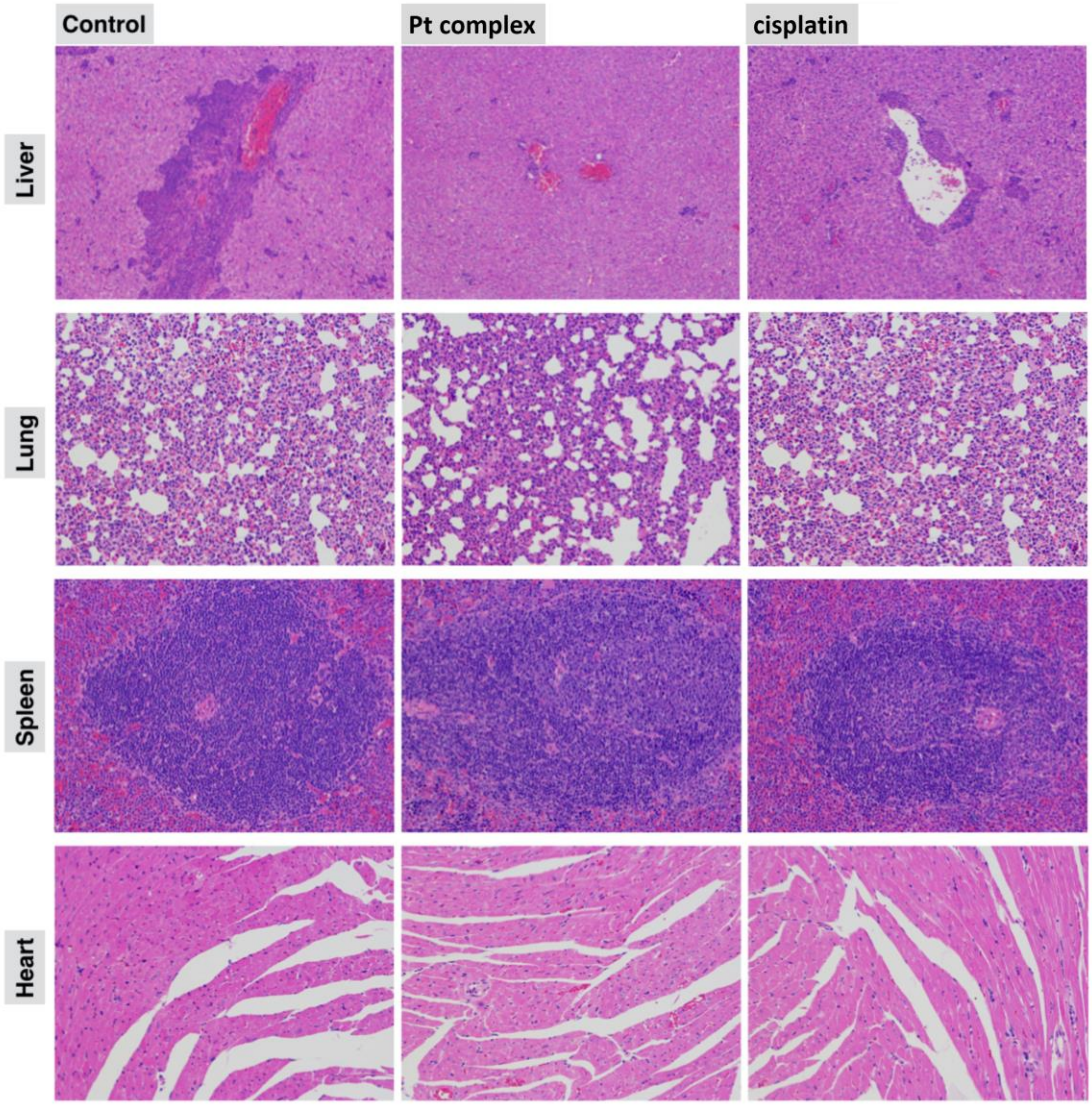


Figure S18. Liver, lung, spleen and heart HE sections from mouse under control, Pt complex and *cisplatin* treated.

Reference

1. Sengupta P, Basu S, Soni S, et al. Cholesterol-tethered platinum II-based supramolecular nanoparticle increases antitumor efficacy and reduces nephrotoxicity. P Natl Acad Sci. 2012; 109: 11294-11299.
2. Li, Qun, Tian Y. Li D. et al. The effect of lipocisplatin on cisplatin efficacy and nephrotoxicity in malignant breast cancer treatment. Biomaterials 2014; 35: 6462-6472.