Electronic Supplementary Information

Mitochondria-Targeted Platinum(II) Complex: Dual Inhibitory Activities on Tumor Cell Proliferation and Migration/Invasion via Intracellular Trafficking of β-catenin

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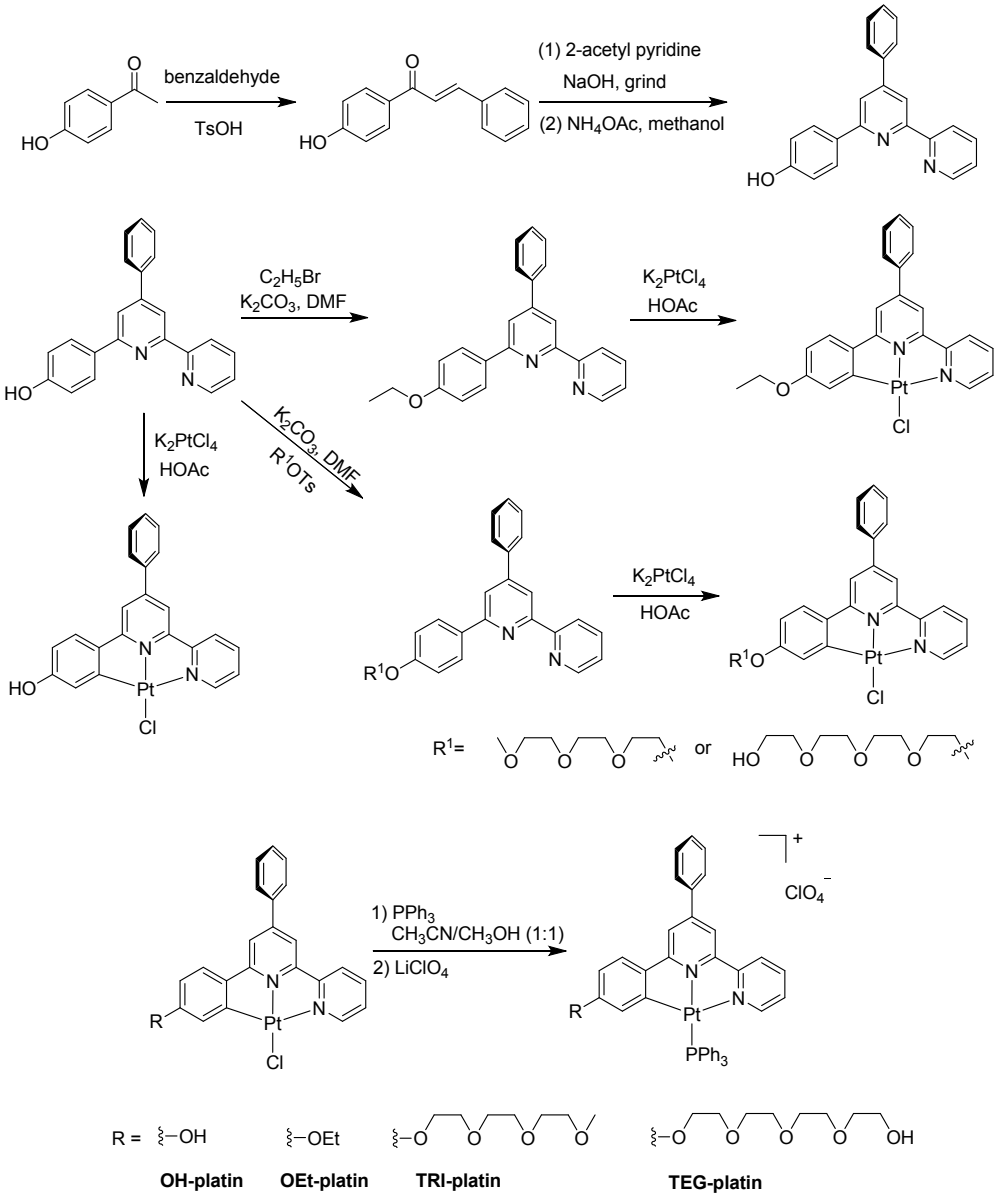
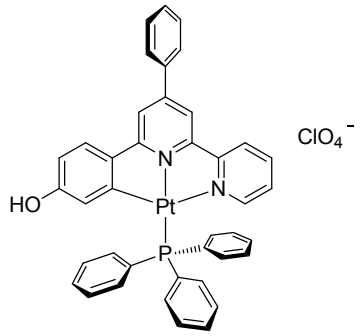
Supplementary Schemes and Figures

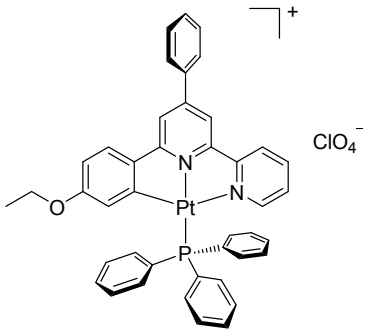


Scheme S1. Synthesis of OH-platin, OEt-platin, TRI-platin, TEG-platin.

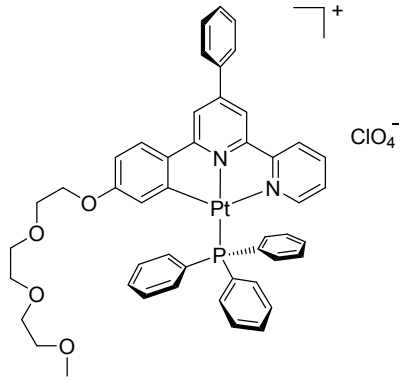
Representative procedure: For all cationinc platinum complex, PPh_3 (0.17 mmol) was added to **[Pt(L^1)Cl]** (0.17 mmol) in CH_3CN/CH_3OH (1:1, 20 mL) and the mixture was stirred for 12 h under a nitrogen atmosphere. The yellow resultant solution was filtered and evaporated to 10 mL. Addition of excess $LiClO_4$ afforded a bright orange solid, which was collected by centrifugation and washed with water (10 mL × 3), ethanol (10 mL × 3) and diethyl ether (10 mL × 3) to give a yellow solid, which was dried *in vacuo*.



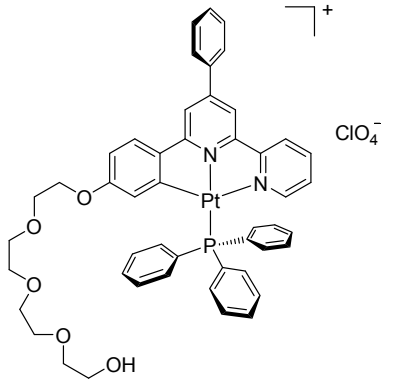
OH-platin: Yield: 100 mg, 70%. ESI-MS (*m*/*z*): 780.2 $[M - ClO_4]^+$. ^1H NMR (CD_3CN, 400 MHz): δ 8.34 (d, J = 8.0 Hz, 1H), 8.17 (m, 1H), 8.08 (m, 1H), 8.04 (m, 1H), 7.97–7.90 (m, 8H), 7.66-7.60 (m, 7H), 7.57–7.53 (m, 6H), 7.08 (m, 1H), 6.63 (m, 1H), 6.49 (m, 1H), 5.90 (m, 1H) ppm. Anal. Calcd for $C_{40}H_{30}ClN_2O_5PPt$: C, 54.58; H, 3.44; N 3.18. Found C, 54.85; H, 3.35; N, 3.30.



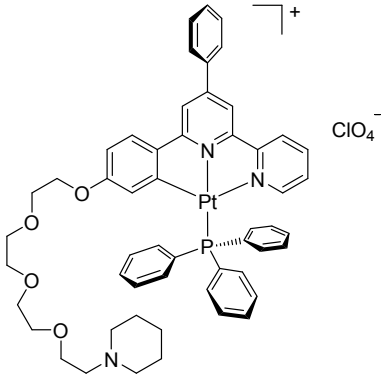
OEt-platin: Yield: 135 mg, 77%. ESI-MS (*m*/*z*): 808.3 $[M - ClO_4]^+$. ^1H NMR (CD_3CN, 400 MHz): δ 8.35 (d, J = 8.0 Hz, 1H), 8.19 (s, 1H), 8.12–8.07 (m, 2H), 7.98–7.90 (m, 8H), 7.68–7.53 (m, 13H), 7.12–7.08 (m, 1H), 6.66 (d, J = 5.2 Hz, 1H), 6.58 (dd, J = 8.8, 2.8 Hz, 1H), 5.98 (m, 1H), 3.23 (q, J = 7.2 Hz, 2H), 0.92 (t, J = 7.2 Hz, 3H) ppm. Anal. Calcd for $C_{42}H_{34}ClN_2O_5PPt$: C, 55.54; H, 3.77; N 3.08. Found C, 55.66; H, 3.85; N, 2.94.



TRI-platin: Yield: 220 mg, 68%. ESI-MS (*m*/*z*): 926.3 $[M - ClO_4]^+$. ^1H NMR (CD_3CN, 400 MHz): δ 8.31 (d, J = 8.0 Hz, 1H), 8.16 (s, 1H), 8.07 (td, J = 8.0, 1.2 Hz, 1H), 8.03 (s, 1H), 7.95–7.89 (m, 8H), 7.67–7.54 (m, 13H), 7.11–7.07 (m, 1H), 6.65 (d, J = 5.2 Hz, 1H), 6.56 (dd, J = 8.0, 2.4 Hz, 1H), 5.95 (m, 1H), 3.53–3.50 (m, 2H), 3.49–3.44 (m, 4H), 3.41–3.39 (m, 2H), 3.33–3.30 (m, 2H), 3.28 (s, 3H), 3.27–3.24 (m, 2H) ppm. ^{13}C NMR (CD_3CN, 100 MHz): δ 164.4, 160.6, 159.4, 155.0, 154.6, 151.6, 141.9, 141.0, 136.9, 136.2, 136.1, 133.2, 133.1, 131.7, 130.2, 130.1, 129.8, 129.2, 128.4, 128.0, 127.8, 125.3, 124.5, 124.4, 113.3, 72.5, 71.1, 70.9, 70.9, 69.3, 67.9, 58.8 ppm. $^{31}P\{^1H\}$ NMR (CD_3CN, 161.8 MHz): δ 25.62 ($^1J_{P,Pt}$ = 4000.5 Hz) ppm. Anal. Calcd for $C_{47}H_{44}ClN_2O_8PPt$: C, 55.00; H, 4.32; N 2.73. Found C, 54.93; H, 4.44; N, 2.65.



TEG-platin: Yield: 115 mg, 65%. ESI-MS (*m*/*z*): 956.4 $[M - ClO_4]^+$. ^1H NMR (CD_3CN, 400 MHz): δ 8.31 (d, J = 8.0 Hz, 1H), 8.16 (s, 1H), 8.07 (td, J = 8.0, 1.2 Hz, 1H), 8.03 (s, 1H), 7.94–7.89 (m, 8H), 7.67–7.54 (m, 13H), 7.10–7.07 (m, 1H), 6.65 (d, J = 6.4 Hz, 1H), 6.56 (dd, J = 8.0, 2.4 Hz, 1H), 5.95 (m, 1H), 4.13–4.10 (m, 2H), 3.61–3.59 (m, 2H), 3.56–3.51 (m, 4H), 3.49–3.47 (m, 2H), 3.41–3.40 (m, 2H), 3.33–3.31 (m, 2H), 3.26–3.24 (m, 2H) ppm. ^{13}C NMR (CD_3CN, 100 MHz): δ 171.5, 164.4, 160.6, 159.4, 154.9, 154.6, 151.6, 141.9, 141.0, 137.3, 137.2, 136.9, 136.2, 136.0, 133.1, 131.7, 130.2, 130.2, 129.8, 129.2, 128.4, 128.0, 127.8, 125.3, 124.5, 117.6, 117.3, 113.2, 71.1, 71.0, 69.5, 69.3, 67.9, 64.2 ppm. $^{31}P\{^1H\}$ NMR (CD_3CN, 161.8 MHz): δ 25.57 ($^1J_{P,Pt}$ = 3998.1 Hz) ppm. Anal. Calcd for $C_{48}H_{46}ClN_2O_9PPt$: C, 54.57; H, 4.39; N 2.65. Found C, 54.85; H, 4.59; N, 2.54.



PIP-platin: Yield: 213 mg, 77%. ESI-MS (*m*/*z*): 1024.4 $[M - ClO_4]^+$. ^1H NMR (CD_3CN, 400 MHz): δ 8.34 (d, J = 8.0 Hz, 1H), 8.19 (s, 1H), 8.09 (t, J = 8.0 Hz, 1H), 8.06 (s, 1H), 7.97–7.90 (m, 8H), 7.68–7.54 (m, 13H), 7.10 (t, J = 6.0 Hz, 1H), 6.66 (d, J = 5.2 Hz, 1H), 6.59 (dd, J = 8.4, 2.4 Hz, 1H), 5.96 (m, 1H), 3.64 (t, J = 5.6 Hz, 2H), 3.58–3.53 (m, 4H), 3.51–3.49 (m, 2H), 3.42–3.40 (m, 2H), 3.34–3.32 (m, 2H), 3.27–3.24 (m, 2H), 2.91–2.87 (m, 6H), 1.69–1.63 (m, 4H), 1.50–1.48 (m, 2H) ppm. ^{13}C NMR (CD_3CN, 100 MHz): δ 163.9, 160.0, 158.9, 156.5, 154.6, 154.2, 151.1, 141.4, 136.4, 135.7, 135.6, 132.6, 131.2, 129.7, 129.6, 129.3, 128.7, 127.9, 127.6, 127.3, 124.8, 117.7, 117.2, 116.9, 112.8, 70.8, 70.4, 70.3, 70.3, 68.8, 67.4, 65.9, 57.4, 54.3, 24.2, 22.7 ppm. $^{31}P\{^1H\}$ NMR (DMSO-d_6, 161.8 MHz): δ 25.53 ($^1J_{P,Pt}$ = 3999.6 Hz) ppm. ^{195}Pt NMR (DMSO-d_6, 85.6 MHz): δ –4143.3 ($^1J_{Pt,P}$ = 4022.3 Hz) ppm. Anal. Calcd for $C_{53}H_{55}ClN_3O_8PPt$: C, 56.66; H, 4.93; N 3.74. Found C, 56.71; H, 4.86; N, 3.58.

Table S1. Cytotoxicity (IC_{50}) of Platinum Complexes and Cisplatin towards six cell lines at 24h

		IC_{50}					
		HepG2	HeLa	MCF7	MRC5	A549	A549/DDP
PIP-platin	μM	8.14±3.47	9.81±2.27	13.63±0.03	28.75±4.18	9.65±0.39	30.50±0.65
	μg/mL	9.14±2.25	11.03±1.47	15.32±0.04	32.32±2.71	10.84±0.25	34.28±0.73
OH-platin	μM	4.62±1.32	14.95±2.05	15.13±1.50	14.32±1.03	10.12±0.70	17.36±3.81
	μg/mL	4.07±0.67	13.16±1.04	13.32±1.32	12.60±0.52	8.90±0.36	15.28±3.35
TRI-platin	μM	3.26±0.56	4.59±1.22	13.49±0.04	4.66±0.08	3.96±0.33	5.14±0.29
	μg/mL	3.34±0.33	4.71±0.73	13.84±0.04	4.78±0.05	4.06±0.20	5.27±0.29
OET-platin	μM	4.49±0.68	10.74±6.30	15.04±0.15	12.10±0.27	4.48±0.38	20.28±4.10
	μg/mL	4.07±0.36	9.75±3.30	13.66±0.13	10.98±0.14	4.06±0.20	18.41±3.72
TEG-platin	μM	4.27±0.30	9.24±5.42	13.56±0.11	9.68±1.37	3.85±0.32	11.12±0.46
	μg/mL	4.51±0.18	9.75±3.30	14.32±0.11	10.22±0.84	4.06±0.20	11.74±0.48
Cisplatin	μM	17.57±3.07	59.22±4.64	58.71±0.07	104.40±17.49	87.70±15.64	230.95±19.64
	μg/mL	5.27±0.53	17.77±0.80	17.63±0.03	31.33±3.03	26.32±2.71	69.31±5.90

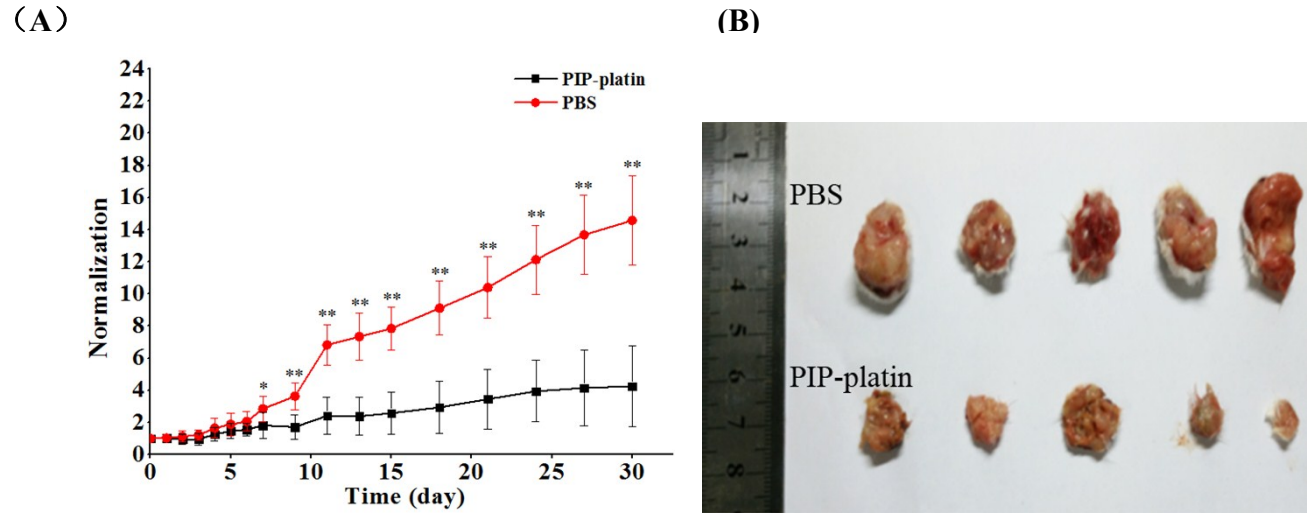


Figure S1. (A) Average tumor volumes of mice bearing 4T1 xenografts after treatment with PIP-platin (15 mg/kg) or PBS vehicle through intratumoral injection. (B) Photograph of *in vivo* tumor volume changes in different groups after various treatment at 30th day. *indicates P<0.05 & **indicates P<0.01 for the difference between PIP-platin and PBS.

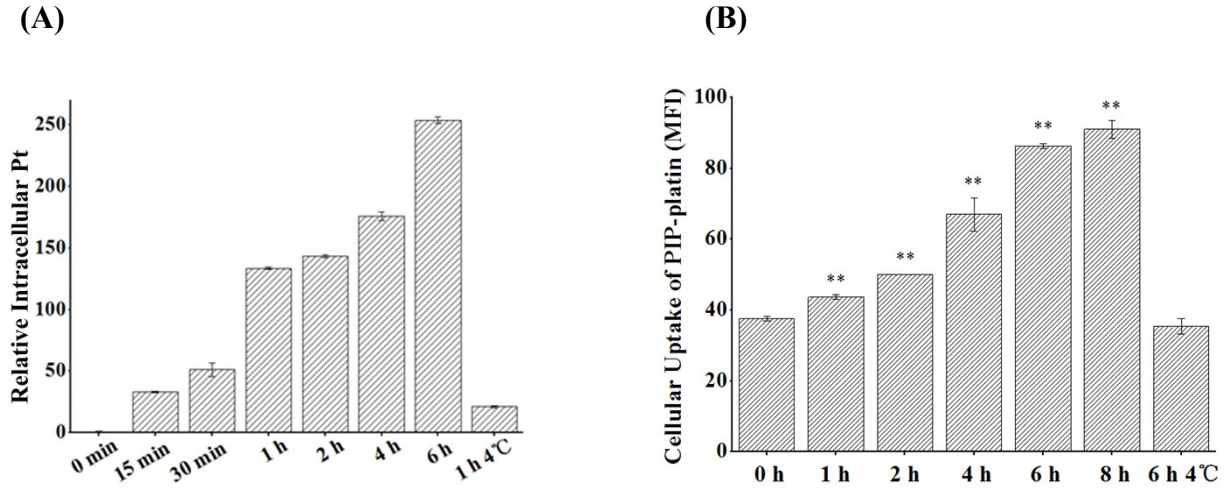


Figure S2. The uptake of PIP-platin was detected using ICP-MS and flow cytometry. (A) The amount of platinum in the cells at different time points were determined by ICP-MS. A549 cells were incubated with PIP-platin at 2.0 μg/mL. "0 min" was set as one fold (cells without any treatment) (B) A549 cells were incubated with PIP-platin at 10 μg/mL at 37°C or 4°C, and the amount of intracellular PIP-platin was determined by flow cytometer with propidium iodide channel (λ_{ex}:488 nm and λ_{em}:610/20 nm).

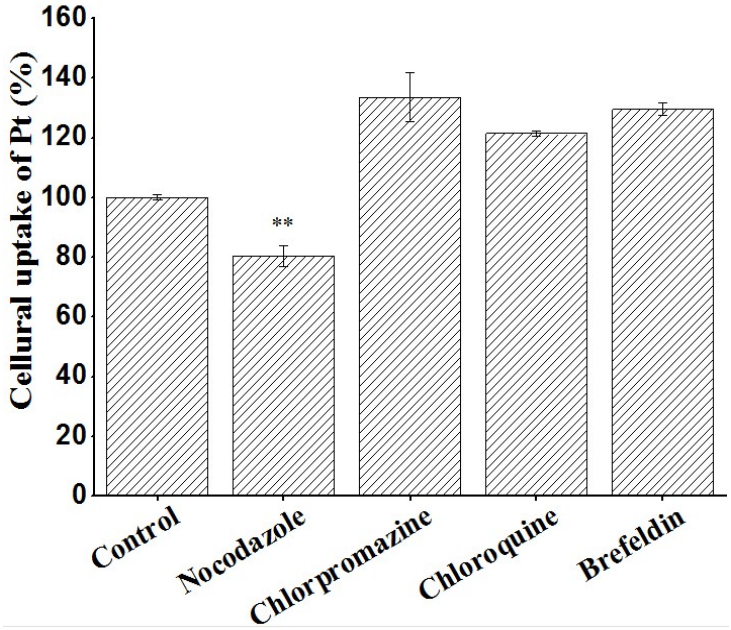


Figure S3. The cellular uptake mechanism of PIP-platin. Cells were pre-treated with endocytic inhibitors such as nocodazole (10 μg/mL), chlorpromazine (10 μg/mL), chloroquine (125 μg/mL) and brefeldin (10 μg/mL) for 30min, followed by PIP-platin incubation for 1 h. The inhibitory effects of these chemicals were normalized to control. **indicates $P<0.01$ for the difference between endocytic inhibitors and control.

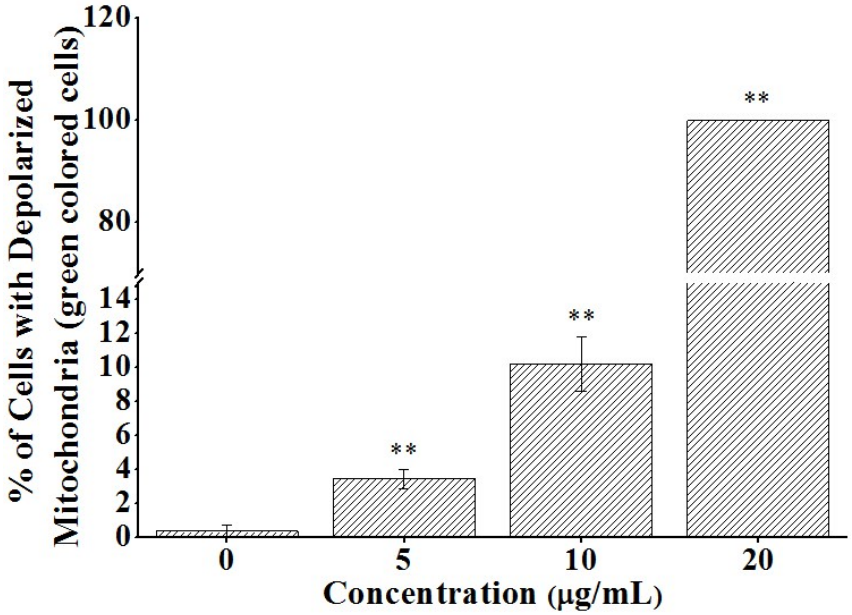


Figure S4. Quantification the effect of PIP-platin on the mitochondrial membrane potential in A549 cells based on Figure 2B. **indicates $P<0.01$ for the difference between PIP-platin and control.

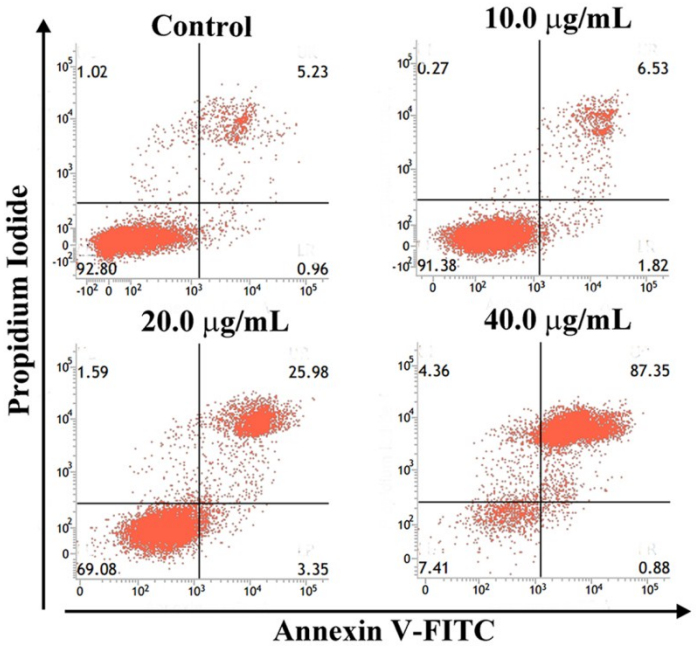


Figure S5. Apoptosis induction by PIP-platin. A549 cells were incubated with control, 10, 20, and 40 μg/mL PIP-platin for 24 h. AnnexinV-FITC/PI Apoptosis Detection Kit was performed, in which red dots from upper right quadrant of the flow cytometry plots indicated necrotic cells and the dots in the lower right quadrant are for apoptotic cells.

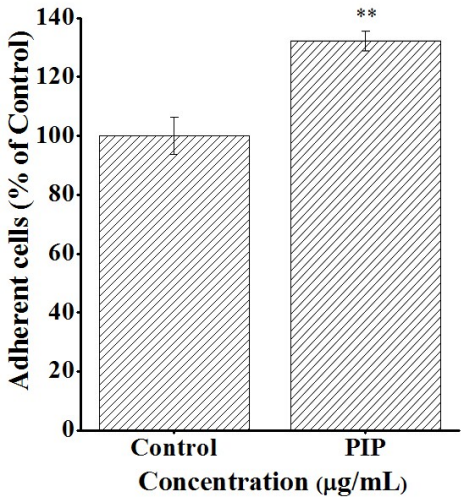


Figure S6. PIP-platin increased cell adhesion. A549 cells were pre-treated with 5 μg/mL PIP-platin, dislodged by trypsinization, seeded into collagen coated 96-well plates. After 1h, non-adherent cells were removed and relative cell numbers were determined by CCK assay. Values represent the means ±

SEM, n=3, ** indicates $P<0.01$ for difference between control and PIP-platin.

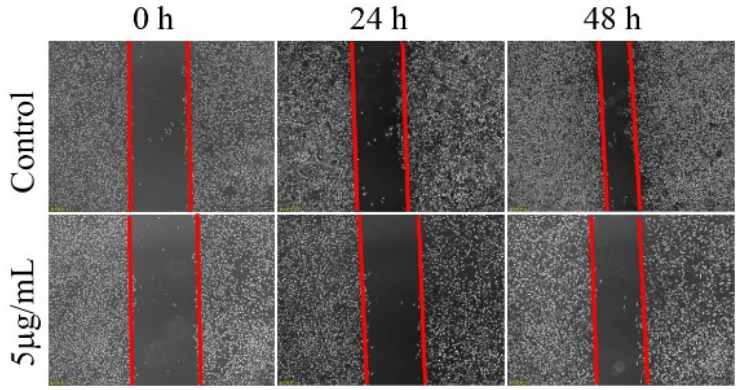


Figure S7. Migration inhibition of MDA-MB-231 metastatic cancer cells by PIP-platin using wound-healing assay. Cells were seeded on 6-well plates until full confluency was obtained. Cells were treated with PIP-platin at 5 μg/mL for 6 h. Cells were scratched and wounded area was recorded at 0 h, 24 h and 48 h. The wounded edge was drawn with red lines. Images are representative of three experiments.

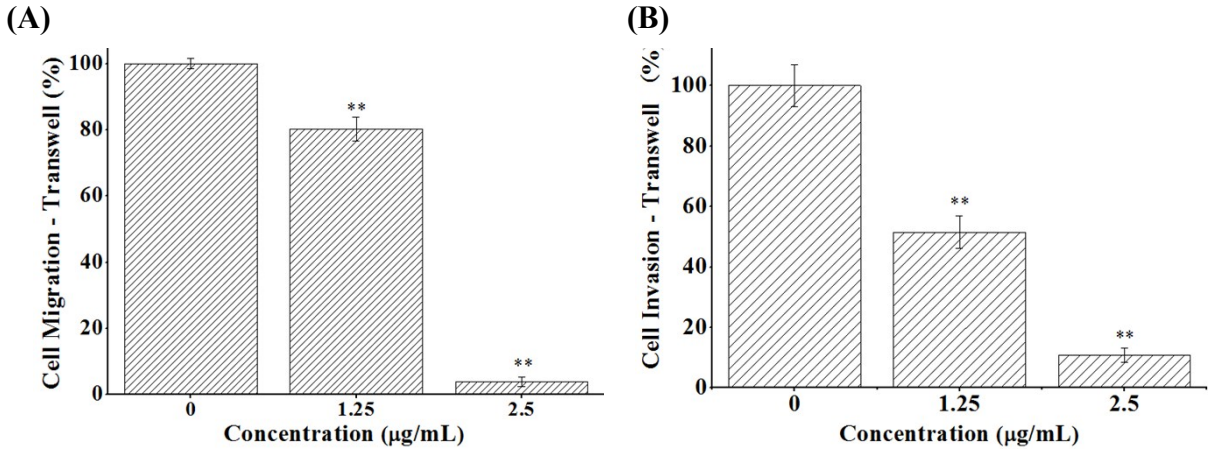


Figure S8. Quantification of inhibition of migration (A) and invasion (B) of metastatic cancer cells (MDA-MB-231) by PIP-platin using transwell assay based on Figure 5C. Data are presented as the number of migrated or invaded cells, values represent the means ± SEM, n=3, **indicates $P<0.01$ for the difference between PIP-platin and control.

Supplementary Materials and Methods

Chemical Synthesis and Characterizations

All experiments were carried out in nitrogen atmosphere unless otherwise noted. Commercial Solvents for synthesis were used without further purification. K_2PtCl_4 (98%) was purchased from Aldrich Chemical Co., The synthesis of platinum complexes were carried out according to our previous work[14]. ^1H, ^{13}C and ^{195}Pt NMR spectra were obtained on Varian YH300 spectrometer and Bruker DRX 400 FT-NMR spectrometers (ppm) using Me_4Si (^1H and ^{13}C) or K_2PtCl_4 (^{195}Pt) as internal standard in deuterated solvents respectively. ESI mass spectra were measured on a Perkin-Elmer SCIEX API 365 LC/MS/MS system. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrophotometer with KBr plates or by the thin film method.

Cell Culture

Human lung adenocarcinoma (A549), human hepatocarcinoma (HepG2), human breast adenocarcinoma (MCF7), human cervical carcinoma (HeLa), normal human fetal lung fibroblast (MRC-5) cell lines, and mouse breast tumor cells (4T1) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science, Shanghai, China. A549cell line was cultured in RPMI1640 culture medium, while the other cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovin serum (Hyclone) and penicillin/streptomycin antibiotics at 37 °C with 5% CO_2 in a humidified atmosphere.

Cell Viability Assay

The cytotoxicity induced by platinum compounds was performed by CCK (Dojindo) assay. Cells were seeded in 96-well plates at a density of $1X10^4$ cells per well (Biofil, Guangzhou, China) overnight, followed by various concentrations of platinum compounds for 24 h. The medium was removed and replaced with 100 μl fresh medium containing 10% CCK for additional 3 h of incubation at 37 °C. The absorbance of each plate was measured with the microplate reader (Synergy NEO, Biotek) at the absorbance wavelength of 450 nm. The cells without any treatment was used as negative controls (fully viable) while cells treated with 1% SDS was set as the fully dead controls that are very close to blank controls without cells. Six wells for each concentration with at least three independent repeats were performed. IC_{50} value was calculated from dose-response curves using Origin 8.0 software.

In Vivo Antitumor Evaluation of PIP-platin

4T1 cells (2×10^6 cells / each mouse) were subcutaneously transplanted into the back flanks of female mice. When the tumors reached a size of ~ 100 mm^3 (about 6 days after transplantation), the mice were randomly divided into 2 treatment groups (solvent control or 15 mg/kg of PIP- platin). Complex PIP- platin (300 □g) was reconstituted in 20 □L of PET diluent (60% polyethylene glycol 400, 30% ethanol, 10% Tween 80) and then dilute to 250 □L with PBS solution. The complexes dissolved in PET diluent and then diluted in PBS or PBS supplemented with equal amount of PET injected into the mice by intratumoral injection everyday (from 0 to 20 days) and every two days (from 21 to 30 days). The tumor sizes and mice weight were measured every day. The tumor volume (V) was calculated as follows: $V = L \times W^2/2$, where W is the tumor measurement at the widest point, and L is the tumor dimension at the longest point. The tumor volumes are normalized against the original volumes at 0 day (V_0) for monitoring the tumor growth. The mice were sacrificed by cervical dislocation under an anesthetic status after the experiments (30 days post-injection). The tumor inhibition was calculated

according to the following formula: Inhibition percentage = $[1-(V-V_0)/(V'-V_0')]\times100\%$, where V and V' are the tumor volumes of PIP-platin treatment and solvent control, respectively. V_0 and V_0' are the initial tumor volumes of the PIP-platin treatment and solvent control, respectively.

Cellular Uptake Studies

The cellular uptake efficiency of platinum compounds was measured by flow cytometric analysis or inductively coupled plasma mass spectrometry (ICP-MS) quantification of Pt. A549 cells were seeded in 6-well plates and treated with 10 □g/mL of PIP-platin (flow cytometry) or 2 □g/mL (ICP-MS) and incubated for different time points at 37 °C or 6 h (flow cytometry) or 1 h (ICP-MS) at 4 °C. The medium was removed and washed three times with PBS to remove residual PIP-platin the medium. Subsequently, the cells were treated with trypsin, centrifuged, washed with PBS and resuspeneded in PBS followed by flow cytometry (FACSVerse, BD) with propidium iodide channel (λ_{ex} = 488 nm, λ_{em} = 610/20 nm). In addition, Pt element in the cells was also quantified by ICP-MS (Thermo ELEMENT X7), of which the detection limit is much more sensitive than PIP-platin fluorescence. To further study the mechanism of uptake, four endocytic inhibitor, such as nacodazole (disrupt microtubule), chlorpromazine (inhibit Rho GTPase), chloroquine (inhibit endosome acidification), brefeldin (interfere with Golgi, endosome and lysosome) were used to dose cells together with PIP-platin and evaluate the uptake inhibition effects by these inhibitors.

Mitochondria Staining and Isolation

A549 cells were seeded in 35 mm plastic dish for 24 h followed by incubation with 2.5 □g/mL of PIP-platin for 2 h followed by incubation with 50 nM Mito-Tracker Green (MTG, Beyotime) for 30 min. Cells were observed for the co-localization between PIP-platin and MTG under Laser Scanning Confocal microscope (UltraViewVoX, PerkinElmer). For mitochondria isolation, A549 cells were treated with 2 □g/mL of PIP-platin for 2 h, mitochondria were isolated by mitochondria isolation kit (Beyotime). The uptake of PIP-platin into the cells and the mitochondria was quantified by determination of the Pt content using ICP-MS. The cellular and mitochondria protein concentration of the same sample was determined by BCA Protein Assay Kit (Beyotime). The amounts of Pt are expressed as nmol Pt per milligram of protein.

Transmission Electron Microscope (TEM)

A549 cells were seeded in 10 cm dish, treated with 5 □g/mL of PIP-platin for 24 h. Following incubation, cells were harvested, washed with PBS. Fixation was performed by Trump's fixative solution overnight at 4 °C, rinsed with 0.1 M PBS and fixed with 1% OsO_4 for 1 h. The cells were dehydrated in a graded series of ethanol and absolute acetone, infiltrated and embedded with Spurr resin. Sections at 70 nm were placed on copper grids, stained with lead citrate and uranyl acetate. The stained samples were imaged with a transmission electron microscope (Tecnai G2 spirit BioTwin, FEI).

Apoptosis Assay

Annexin-V/Propidium iodide (PI) Kit (Beyotime) was used for apoptotic cell detection. Annexin-V has a high binding affinity for the membrane phospholipid phosphatidylserine, translocated from the inner to outer of plasma membrane during apoptosis, while PI can stained imperfect membrane of necrosis and late apoptosis cells. A549 cells were plated in 24-well plates and treated with PIP-platin for up to 24h. After treatment, cell were stained with Annexin-V and PI for 30 min and measured by flow

cytometer.

Intracellular ROS Measurement

The production of ROS was assessed by DCFH-DA (Beyotime), a cell-permeable nonfluorescent probe, which can penetrate into cell and upon oxidation it turns to a highly fluorescent dye. A549 cells were seeded in 96-well plates. After treatment with PIP-platin, the cells were loaded with DCFH-DA at 10 □M for 20 min at 37 °C in the dark. Subsequently, the medium was removed and washed with serum-free medium. The fluorescent intensity of cells was monitored by flow cytometer.

Mitochondrial Membrane Potential (MMP) Determination

MMP was measured using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl- carbocyanine iodide (JC-1, Beyotime), which can accumulation in mitochondria, being converted by a fluorescence emission shift from red (590 nm) to green (525 nm) due to the mitochondrial depolarization and is indicated by a decrease in the red/green fluorescence intensity ratio. A549 cells were seeded on 35 mm cell culture dishes and incubated with PIP-platin followed by the staining with JC-1 staining solution for 30 min at 37°C. The cells were detected and visualized by Laser Scanning Confocal microscopy and the percentage of cells with depolarized mitochondrial membrane was determined by three independent images for each sample.

Intracellular ATP Detection

To estimate the intracellular level of ATP, ATP Assay Kit (Beyotime) was used according to the manufacturer's instructions. In brief, cells were seeded into 24-well cell culture plates, incubated with or without PIP-platin or cisplatin, lysed with 400 □L ice-cold ATP-releasing buffer, centrifuged at 12,000 g for 10 min, washed with PBS three times. ATP detection buffer was added and luminescence was measured by a plate reader (Synergy NEO, Biotek). The intracellular level of ATP was calculated and normalized to control.

Immunoprecipitation and Western Blot

Cells were washed with cold PBS, and the intracellular protein were released with RIPA lysis buffer supplemented with protease inhibitors cocktail (Sigma), 1 mM DTT, 1 mM Na3VO4, 10 mM NaF, 1 mM PMSF, and 1 mM □-Glycerophosphate. The cell suspension was incubated on ice for 10 min and centrifuged at 14,000 g at 4 °C for 15 min. The supernatant was collected and the protein concentrations were measured using BCA Protein Assay Kit. Cell lysates were denatured at 99 °C and resolved by SDS-polyacrylamide gel electrophoresis. Total cell lysates were analysed by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, visualized using a commercially available enhanced chemiluminescence kit (Thermo).

Immunofluorescence Staining

Cells were seeded on cover slips in 24-well plates, treated with PIP-platin, washed with PBS, fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with 0.1% triton, blocked with 5% normal goat serum and incubated overnight at 4°C with rabbit anti-Cytochrome c, or β-catenin followed by Alexa Fluor® 568 conjugated goat anti-rabbit immunoglobulin secondary antibody for 1 h. For □-catenin staining, the cells were serum starved overnight and fixed followed by primary antibody incubation and Alexa Fluor® 568 conjugated secondary antibody staining. The stained samples were

observed with inverted fluorescence microscope.

Trypsinization Assay and Cell Adhesion Assay

A549 cells were treated with PIP-platin for 6 h, and 0.15% trypsin was used to trypsinize the cells for 1, 2, 3, 4 min at 37°C. Trypsinization was terminated by addition of serum-containing medium and CCK-8 was used for detection of cell viability (relative cell number). Cell adhesion assay was performed as the following procedure: collagen gels at 5 □g/mL were immobilized on 96-well plates in PBS for overnight at 4°C. The wells were washed with PBS, blocked with a 0.5% BSA solution in PBS for 1 h and washed three times with PBS. PIP-platin and cisplatin pretreated cells (6 h) were seeded at 500 mL/60 K/well on the coated wells and incubated at 37°C for 1 h. After incubation, the wells washed with PBS to remove unattached cells, and the percentage of adherent cells were determined by CCK assay.

Wound-healing Assay and Transwell Migration/Invasion Assays

Cell migration was analyzed by wound-healing assay by using MDA-MB-231 cells. Cells were seeded into 6-well dishes and allow to proliferate to almost total confluence. Wounds were made by scratching with a sterilized one-milliliter pipette tip across the middle of the cell monolayer. Cells were cultured in NIH-3T3 cell conditioned medium. NIH-3T3 fibroblast cell conditioned medium provided cytokines and chemoattractants essential for MDA-MB-231 cells to migrate into the transwell network. Migration of cells toward to the wound area was visualized under the inverted microscope at 24 h and 48 h. Transwell migration/invasion was performed using 24-transwell inserts (Corning). After incubated with or without PIP-platin for 24 h, MDA-MB-231 cells (50,000 cells/well) were suspended in 200 □L of serum-free medium and added into the upper chamber, and migration inducer (NIH-3T3 cell conditioned medium) was added into the lower chamber. Invasion assays were performed similarly except for placing cells on Matrigel (Invitrogen) in upper chamber. Cells that migrated and invaded through the membrane were stained with Giemsa and quantified, and normalized to control.

Wnt3a Protein Preparation

HEK293T (Human embryonic kidney cells) cells were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO_2 incubator. For Wnt3a purification, Wnt3a-producing Schneider2 (S2) cells, a Drosophila cell line, were cultured in capped flasks at 23□25 °C with no CO_2, in 1 × Schneider's Drosophila Medium (Invitrogen), 10% FBS (heat inactivated, insectcell tested, from Sigma), 1 × PSQ and scaled up to get desired mount of conditioned medium. The Wnt3a was purified by FPLC.

Wnt Reporter Assay

HEK293T were seeded in 24-well Cell Culture Cluster (Corning) and transfected with Topflash reporter and GFP plamids using Lipofectamine 3000 Transfection Reagent (Life Technologies) according to the manufacturer's instructions. Topflash is a luciferase reporter plasmid containing TCF binding regions. If the Wnt signaling is activated, β-catenin will translocate to the nucleus to bind the TCF/LEF transcription factors and activate the transcription of Wnt target genes with the increased luciferase activity. After 20 h of transfection, medium replaced with control or Wnt3a containing medium for additional 6 h. The cells were lyszed and the lysate was used for luciferase activity (indicative of Wnt activity) and fluorescence intensity (indicative of transfection efficiency) assessment. The relative luciferase units (RLU) was acquired by normalizing luciferase activity to the fluorescence.

Membranous, Cytosol and Nuclei Fractionation

HEK293T at 1~2×10^7 were scraped with PBS into 1.5 mL microtube, centrifuged at 1800 g for 5 min. After washed with buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors), the pelleted cells were resuspended with 100 □L buffer A and incubated on ice for 10 min. Cells were then passed through a 0.4-mm needlepoint to break down the cell membrane and were then centrifuged at 3300 g at 4 °C for 10 min. The supernatant was collected and centrifuged at 100,000 g at 4 °C for 1 h. The supernatant from the ultracentrifugation was collected as the cytosolic fraction and pellet was dissolved with equivalent volume of 2 × SDS loading as the membranous fraction. For nuclear protein extraction, the pellet from the 3300 g centrifugation was washed once with buffer A, resuspended in buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.2 mM PMSF and protease inhibitors) and then incubated on ice for 30 min with shake every 3 to 5 min. After centrifugation at 100,000 g at 4 °C for 1 h, the supernatants were collected as nuclear extracts.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical significance was calculated by one-way analysis of variance followed by Turkey pos-thoc test to determine the difference between groups. * indicative $0.01<P<0.05$ and ** indicative $P<0.01$ was considered to be statistically significant.