Electronic Supplementary Material (ESI) for Metallomics.

Electronic Supporting Information

for

Aspirin Ligation Enhances Drug Efficacy by Altering the Cellular Response

Content

Experimental details

Materials. *cis*-[$PtCl_2(NH_3)_2$] (cisplatin) was purchased from sigma. *cis*, *trans*, *cis*-[$PtCl_2(OH)_2(NH_3)_2$] (oxoplatin) was synthesized according to the literature (*J. Biol. Inorg. Chem.* 8(7):726-732). Asplatin was synthesized according to our previous method (*Chem. Commun.*, 2014, 50, 7427-7430). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Cytoskeleton Inc. (Denver, USA). Ultrapure water (18.2 MΩ) from Millipore Milli-Q Biocel purification system containing a 0.22 μm filter was used for all experiments.

Phosphate buffered saline (PBS) contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4. The solution was adjusted to pH 7.4. Cell lysis buffer for western blot contains 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA and 50 mM HEPES at pH 7.5.

Animals. NOD/SCID mice (6 weeks old) were purchased from the Beijing Vital River Bioscience Co., Ltd (Beijing, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee.

Cell culture. The human cancer cells, including cervical HeLa, breast carcinoma MCF-7, hepatocellular carcinoma HepG2, lung carcinoma A549 cells were obtained from the American Type Culture Collection (ATCC). The cells were maintained in either DMEM (for HeLa, MCF-7 and HepG2 cells) or RPMI1640 (for A549 cells) medium containing 10% fetal bovine serums in a humidified atmosphere containing 5% CO_2 at 37°C.

Cell cytotoxicity assays. HeLa cells were seeded in 96-well plates at 5000 cells per well in 100 μL of complete medium, and incubated in a 5% CO_2 atmosphere at 37°C for 24 h. The culture medium was then replaced with 100 μL of freshly prepared culture medium containing drugs at different concentrations. The cells were further incubated for 24 h, and then the medium was replaced with fresh culture medium and the MTT solution was added. The cells were incubated for another 3 h to allow viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, 100 μL of lysis buffer was added to wells and incubated for another 4 h at 37°C. The absorbance was measured at 570 nm using a Bio-Rad 680 microplate reader. The IC_{50} values were calculated using GraphPad Prism software (version 5.01) based on data from three parallel experiments.

Cellular platinum accumulation. For quantification of the cellular platinum accumulation, HeLa cells were seeded in 6-well plates overnight and then incubated with various dosage platinum compounds at 37℃ in the standard culture condition for 3 h. Then the cells were washed with PBS there times, and harvested by trypsinization. The harvested cells were digested with nitric acid for the ICP-MS measurement. The cell numbers were counted before the digested.

Cell cycle analysis. HeLa cells were seeded at a density of 10×10^4 cells per well in 1 mL DMEM medium and incubated in a humidified 5% CO_2 atmosphere for 24 hours. The original medium was replaced with fresh medium containing 4 μM drugs for 24 or 48 h. After then the cells were harvested for DNA content analysis using the CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences, San Jose, USA), according to the manufacturer's instructions. Cell cycle distributions and DNA contents were determined using BD FACS Calibur Flow Cytometer. The results were analyzed using FlowJo software. Percentages of cells in G1, S, G2/M phases were recorded.

Apoptosis analysis with AnnexinV/PI assay. HeLa cells cultured in 12-well plates were treated with 4 μM drugs for 24, 48 and 72 h. Apoptotic cells were detected by flow cytometry after staining with Annexin V and Propidium Iodide (PI) using the AnnexinV-FITC apoptosis detection kit I (BD Biosciences, San Jose, USA). The data were analyzed using FlowJo software.

Real-Time PCR. Total cellular RNAs extracted from cells with RNAiso Plus (Takara, Dalian, China) were reverse transcribed into cDNA using PrimeScript® RT reagent Kit (Perfect Real Time) (Takara, Dalian, China) according to the manufacturer's instructions. After then, 1 μL of cDNA was subjected to quantitative real-time PCR analysis interest genes and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the SYBR Premix Ex Taq (Perfect Real Time) (Takara, Dalian, China). The assay was performed on an Applied Biosystems StepOne Real-Time PCR Systems. The relative gene expression values were determined by the ΔΔCT method

using StepOne Software v2.1 (Applied Biosystems). Data were presented as the fold differences in interest genes normalized to the housekeeping gene *GAPDH* as the endogenous reference and relative to the untreated control cells. The primers used in the quantitative real-time PCR were listed in Table S1.

Western blot analysis. For the Western blot analysis, HeLa, HepG2 and MCF-7 cells were treated with drugs for 24 h and washed twice with cold PBS, and then resuspended in 50 μL of RIPA lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM $MgCl_2$, 1 mM EDTA). Cell lysates were incubated on ice for 30 min and vortexed every 5 min. The lysates were centrifugalized at 12000 g for 10 min to obtain the whole cell fraction. For obtaining the cytoplasmic protein, cells were resuspended in 100 μL homogenizing buffer (250 mM Sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT) with a mixture of protease inhibitors. After 30 min of incubation on ice, the lysates were centrifugalized at 12000 g for 20 min to obtain the cytoplasmic fraction. Protein concentration was determined using a BCA Protein Assay Kit (lot: 23250, Thermo, Madison, WI). Total protein (40 μg) was separated on 10% or 12% Bis-Tris-polyacrylamide gels and then transferred to Immobilon-P membranes (Millipore, Bedford, USA) at 300 mA for 30-60 min. After incubation in 5% BSA in PBST for 1 h, the membranes were incubated with the appropriate primary antibody (anti-P53, 1:2000, sc-126; anti-β-actin, 1:10000, sc-130301; anti-BCL-2, 1:1000, sc-7382; anti-cytochrome c, 1:2000, sc-13156; Santa Cruz Biotech., Santa Cruz, USA; anti-Caspase 9, 1:2000, #9502; anti-cleaved PARP, 1:2000, #5625; Cell Signaling Technology, Inc.) overnight at 4°C. The membrane was further incubated with the appropriate secondary antibody (anti-mouse IgG, 1: 10000, sc-2005; anti-rabbit IgG, 1:10000, sc-2004; Santa Cruz Biotech., Santa Cruz, USA) for 60 min at room temperature and visualized using the ECL system (Pierce, Rockford, USA). Expression levels of interest proteins were normalized against β-actin protein expression levels.

TMRM Assay. HeLa and HepG2 cells were cultured on cover slips to a density of 1×10^5 cells/mL and incubated overnight at 37°C. Cells were then treated with 4 μM or 6 μM drugs for 18 h at 37°C. A solution of TMRM reagent (ImmunoChemistry Technologies, USA) was added to a final concentration of 2 μM and incubated at 37°C for 20 min. For fluorescence imaging, the cells were washed with PBS five times, and fixed in 4% paraformaldehyde. The nuclei were stained with DAPI (blue, Sigma-Aldrich, St. Louis, MO, USA). After then the cells were mounted onto glass slides using mounting solution for imaging under a LSM 710 CLSM (Carl Zeiss, Jena, Germany) using a ×40 objective and excitation wave lengths of 548 nm and 405 nm for TMRM (red) and DAPI (blue), respectively. For fluorescence density analysis, the TMRM stained cells were washed with PBS five times and collected for analyses using BD FACSCalibur Flow Cytometer.

Histology, immunohistochemistry and in situ cell death detection. After the *in vivo* antitumor assay in our previous reported (*Chem. Commun.*, 2014, 50, 7427-7430), the tissues were removed and fixed in 4% paraformaldehyde, paraffin-embedded, sectioned into 5 μm sections and mounted onto poly-L-lysine-coated glass slides, at the First Affiliated Hospital of Anhui Medical University. The slides were stained with haematoxylin/eosin (H&E). The proliferation of tumor cells was detected using an antibody against PCNA. Deparaffinized slides were boiled for 5 min in 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker for antigen retrieval. Subsequently, slides were allowed to cool for another 5 min in the same buffer. After several rinses in PBS and pretreatment with blocking medium for 5 min, slides were incubated with the PCNA antibody (Maxim Biotech., Fuzhou, China) diluted to 1:200 in antibody diluent solution for 60 min at 37°C, followed by incubation with a secondary antibody kit (ZhongShan Golden Bridge Biotechnology, Beijing, China) for 15 min at 37°C. After being treated with DAB reagent (ZhongShan Golden Bridge Biotechnology), the slides were counterstained with hematoxylin. Apoptotic levels in tumor or kidney cells following the various treatments were also determined using the transferase-mediated dUTP nick end-labeling (TUNEL) method according to the manufacturer's instructions (In Situ Cell Death Detection Kit, POD; Roche, Basel, Switzerland). All slides were examined under an Olympus IX81 microscope (Tokyo Prefecture, Japan).

Statistical Analysis. The statistical significance of treatment was assessed using the Prism software (GraphPad); The statistical differences were determined by Tukey's Multiple Comparison Test. Values $p < 0.05$ indicate significant differences.

Table S1. The primers used in the quantitative real-time PCR

Survivin-FW	5'-GACCACCGCATCTCTACAT-3'	Survivin-RV	5'-CTTGGCTCTTTCTCTGTCC-3'
BCL-2-FW	5'-ATGTGTGTGGAGAGCGTC-3'	BCL-2-RV	5'-CCAAACTGAGCAGAGTCTTC-3'
P53-FW	5'-TGCTCAGATAGCGATGGTCT-3'	P53-RV	5'-CACAAACACGCACCTCAAAG-3'
BCL-xL-FW	5'-GAAAGCGTAGACAAGGAGATG-3'	BCL-xL-RV	5'-AAGAGTGAGCCCAGCAGAA-3'
BAD-FW	5'-CAACCAGCAGCAGCCATCAT-3'	BAD-RV	5'-ATCCCTTCGTCGTCCTCCGT-3'
BAX-FW	5'-ATGCGTCCACCAAGAAGC-3'	BAX-RV	5'-CCCAGTTGAAGTTGCCGT-3'
BAK-FW	5'-CGACATCAACCGACGCTATG-3'	BAK-RV	5'-CCACTCTCAAACAGGCTGGTG-3'
VEGF-FW	5'-CCTGGTGGACATCTTCCAGGA GTACC-3′	VEGF-RV	5'-GAAGCTCATCTCTCCTATGTGCTGGC-3'
GAPDH-FW	5'-TCCACCACCCTGTTGCTGTA-3'	GAPDH-RV	5'-ACCACAGTCCATGCCATCAC-3'

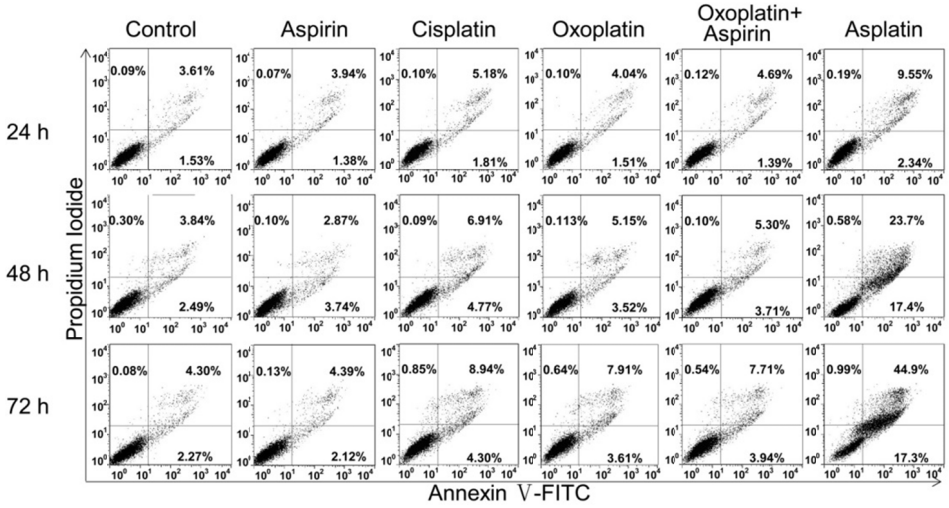


Figure S1. Time dependent apoptosis induced by platinum complexes. HeLa cells were treated with 4 μM platinum complexes or aspirin for different time (24, 48, 72 h). Cells were stained with Annexin V/PI and analyzed with flow cytometry.

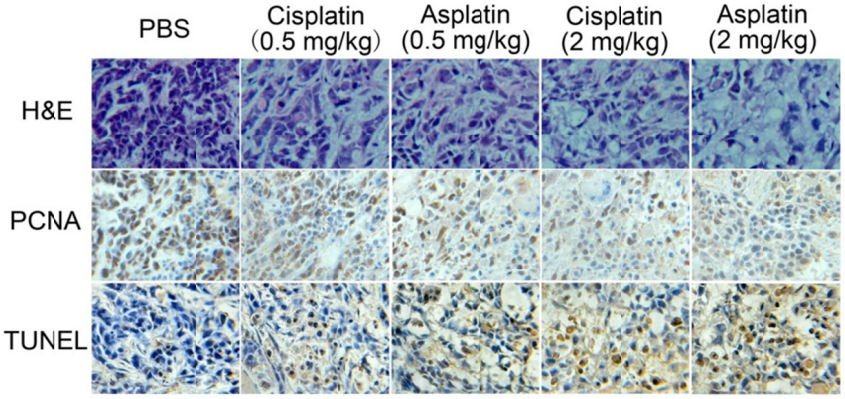


Figure S2. Immunohistochemistry analyses on tumor tissues. NOD/SCID mice bearing HepG2 tumors were treated with cisplatin or asplatin (n = 8 at dose 0.5 or 2 mg Pt/kg, q3d × 5). PBS was used as control. H&E stain: hemalum stains nuclei in blue and eosin Y stains eosinophilic cytoplasm in various shades of red, pink and orange. The PCNA-positive proliferating cells and TUNEL apoptotic cells are stained brown.

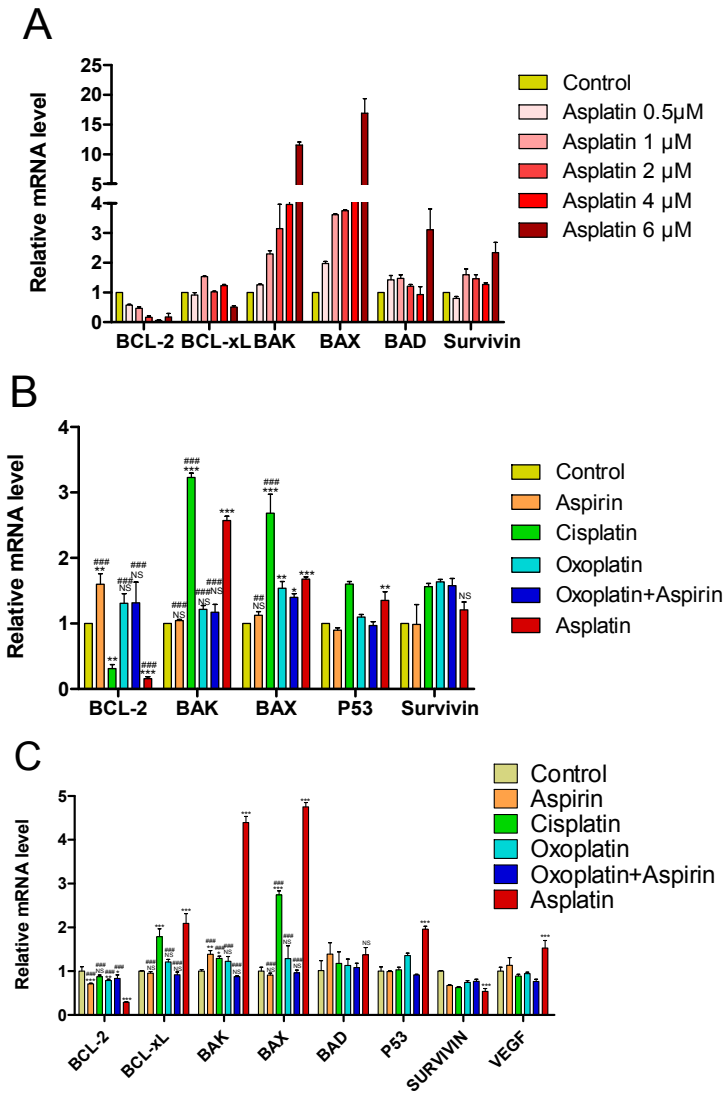


Figure S3. RT-PCR analyses of the expression of apoptosis-related genes. (A) Incubation of HeLa cells with various concentrations of asplatin. (B) Incubation of A549 cells with 6 μM different compounds. The mRNA level was analyzed by Real-time PCR after 24 h incubation. (C) Incubation of MCF-7 cells with 8 μM different compounds. The mRNA level was analyzed by Real-time PCR after 24 h incubation. Error bars denote standard deviations of three independent experiments, asterisks indicate p-values versus control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) and NS means 'Not Statistically Significant' compared to control. Pound signs indicate p-values versus asplatin treatment (#: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$).

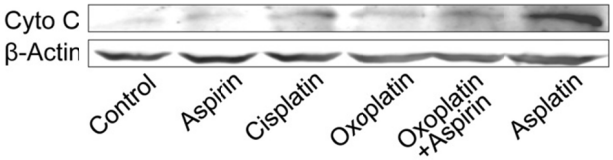


Figure S4. Effects of asplatin on the release of cytochrome c on MCF-7 cells. MCF-7 cells were treated with 8 μM platinum complexes. Western blotting was performed after 24 h incubation.

Table S2. Inhibitory effect (IC_{50} in μM) of asplatin on HeLa cells after 24 h incubation.

cisplatin	cisplatin + aspirin	oxoplatin	oxoplatin + aspirin	asplatin
21 ± 2	20 ± 1	78 ± 5	75 ± 3	4.2 ± 0.6

Table S3. The gene expression level in HeLa cells treated with platinum complexes in the concentration of IC_{50}.

		BCL-2	BAK	BAX	BCL-xL
Control		1.00±0.01	1.00±0.01	1.00±0.06	1.02±0.23
Cisplatin	(20.9 μM)	0.44±0.04	1.56±0.10	1.96±0.28	1.19±0.17
Cisplatin+Aspirin	(20.5 μM)	0.44±0.03	1.52±0.05	1.92±0.23	1.33±0.35
Oxoplatin	(78.1 μM)	0.23±0.02	1.96±0.19	2.14±0.04	1.00±0.05
Oxoplatin+Aspirin	(75.0 μM)	0.25±0.01	2.18±0.15	2.32±0.06	1.24±0.06
Asplatin	(4.23 μM)	0.024±0.01	5.04±0.63	3.37±0.61	0.57±0.08

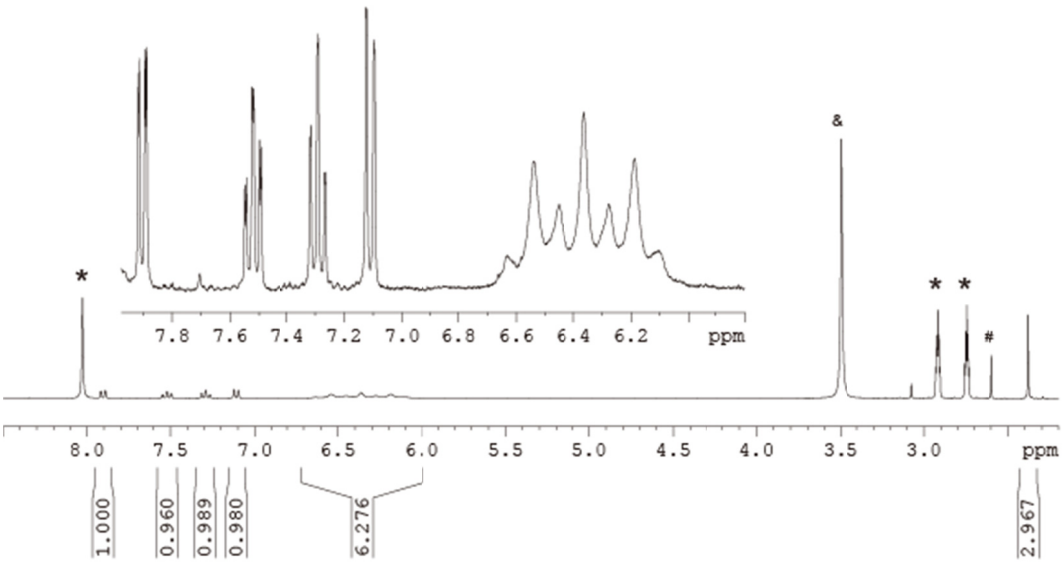
* Data are plotted in Figure S5B.

Table S4. The gene expression level in HeLa cells treated with platinum complexes in the concentration of the same cellular uptake.

		BCL-2	BAK	BAX	BCL-xL
Control		1.00±0.08	1.00±0.05	1.00±0.02	1.00±0.07
Cisplatin	(25 μM)	0.26±0.04	2.27±0.37	2.49±0.06	0.98±0.08
Cisplatin+Aspirin	(25 μM)	0.30±0.07	2.24±0.40	2.53±0.04	0.88±0.05
Oxoplatin	(60 μM)	0.37±0.01	2.03±0.16	1.94±0.15	1.08±0.02
Oxoplatin+Aspirin	(60 μM)	0.34±0.01	1.96±0.07	1.93±0.15	1.01±0.04
Asplatin	(5 μM)	0.016±0.01	5.78±0.46	4.16±0.42	0.47±0.01

* Data are plotted in Figure S5D.

(A) ^{1}H NMR in d^{7}-DMF



(B) ^{195}Pt NMR in d^{7}-DMF

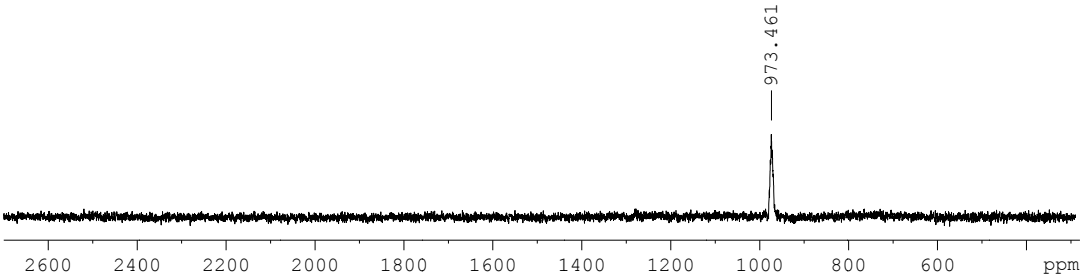


Figure S6. NMR characterization of asplatin (*c,c,t*-[Pt(NH_3)$_2$Cl$_2$($C_9H_7O_4$)OH]). (**A**) ^{1}H NMR spectrum of asplatin in d^{7}-DMF. The inset shows the expansion of NH_3 and aromatic proton signals. The NH_3 signal appears at ~ 6.36 ppm. Residue solvent peaks are labeled in the spectrum: DMF (*) 8.03 ppm, 2.92 ppm and 2.75 ppm; H_2O (&) 3.5 ppm; DMSO (#): 2.59 ppm. (**B**) ^{195}Pt NMR spectrum of asplatin in d^{7}-DMF.