Electronic Supporting Information for

A Carrier-free Nanostructure Based on Platinum(IV) Prodrug Enhances Cellular Uptake and Cytotoxicity

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Content

Materials and instruments.

Cisplatin was purchased from Dalian Meilun Biological Technology Co. Ltd. Flurbiprofen was purchased from J&K Chemicals. Oxalyl chloride, ascorbic acid (AsA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2'-deoxyguanosine 5'-monophosphate sodium salt hydrate (5'-GMP) were purchased from Sigma Aldrich. All chemicals were analytic grade and used without further purification. Ultrapure water purified by Millipore Milli-Q Biocel purification system (18.2 MΩ) containing a 0.22 μm filter was used for all experiments.

^{1}H and ^{13}C-NMR spectra were registered on a Bruker AVANCE 400 spectrometer (USA). ^{195}Pt NMR spectrum was recorded on a Bruker AVANCE 600 spectrometer (USA). ESI-MS spectra were measured on a Thermo scientific Q-Exactive instrument (Germany). HPLC analyses were made on a Shimadzu LC-20 instrument (Japan) equipped with a multi-wavelength UV-visible detection.

Synthesis of *c, c, t*-$Pt(NH_3)_2Cl_2(OH)_2$

Hydrogen peroxide (30% w/v, 4 ml) was added to the suspension of cisplatin (507.09 mg, 1.69 mmol) in water (10 ml). The reaction mixture was stirred for 3 h at 50 ℃ in dark and stored at 4 ℃ overnight. Then the bright yellow crystals $Pt(NH_3)_2Cl_2(OH)_2$ was separated by filtration, washed with cold water and ethanol, dried in vacuum and stored in dark at room temperature. Yield: 71%.

Synthesis of platin-FP

Flurbiprofen (801.97 mg, 3.28 mmol) and oxalyl chloride (4 ml) were mixed and refluxed for 1 h at 70 ℃. Excess oxalyl chloride was removed by rotary evaporation. Dry THF (5 ml) was added and evaporated. Repeat the above step to remove oxalyl chloride completely. A yellow oil-like flurbiprofen chloride was obtained.

$Pt(NH_3)_2Cl_2(OH)_2$ (54.85 mg, 0.16 mmol) was mixed with the freshly prepared flurbiprofen chloride in dry THF (6 ml) and refluxed for 2 h at 70 ℃. After removal of THF under vacuum, acetone (5 ml) and water (25 ml) were added in turn. The

mixture was stored at 4 ℃ overnight. Acetone was removed by rotary evaporation. Then the aqueous solution was decanted and the residue was dried in vacuum. The crude product was resuspended in cold diethyl ether and stored at 4 ℃ for 4 h. The light yellow product was separated by filtration, washed with cold diethyl ether, dried in vacuum and stored in dark at room temperature. Yield: 87%.

Electrochemistry

Electrochemical measurements were made at 25 ℃ on a CHI 660C electrochemical workstation (China) equipped with a three-electrode set-up comprising a glassy carbon working electrode, platinum wire auxiliary electrode and an Ag/AgCl reference electrode. The electrochemical data were uncorrected for junction potentials. $(C_4H_9)_4NClO_4$ (0.1 M) was used as a supporting electrolyte. Platin-FP and $(C_4H_9)_4NClO_4$ were dissolved in DMSO-H_2O (v/v, 3:1). The final concentration of platin-FP is 1 mM.

The reduction of Platin-FP *in vitro*

Platin-FP (1 mM) and ascorbic acid (AsA, 10 mM) were dissolved in DMSO/water (3:1, 4 ml) and co-incubated for 48 h at 37 ℃. Platin-FP without ascorbic acid was served as a control. The reduction of platin-FP was monitored by HPLC at different time points. Elution A was H_2O (0.1% TFA), and elution B was methanol. The linear gradient was varied from 70% to 100% elution B in 5 – 15 mins. The flow rate was 1.0 ml/min and the UV detection wavelength was 245 nm.

Detection of Pt-GG *in vitro*

Platin-FP (1 mM), 2’-deoxyguanosine 5’-monophosphate sodium salt hydrate (5’-GMP, 5 mM) and ascorbic acid (AsA, 10 mM) were dissolved in DMSO/water (3:1) and co-incubated for 36 h at 37 ℃. The mixture was analyzed by ESI-MS.

***In vitro* COX-2 inhibition assay**

The ability of platin-FP and flurbiprofen to inhibit COX-2 was assayed by a COX-2

inhibitor screening assay kit (Beyotime, China). Celecoxib (1 μM) was used as the positive control. Samples and reagents were prepared according to the instruction. The test procedure was performed following the manufacturer protocol.

Cell culture

Human colon cancer cell line SW480, human prostate cancer cell line PC-3 and human pancreas cancer cell line PANC-1 were obtained from the ATCC. Human lung cancer cell line A549 and its cisplatin-resistant cell line A549-DDP, human liver cancer cell line BEL7404 and its cisplatin-resistant cell line BEL7404-CP20 were generously gifted by Michael M. Gottesman's laboratory at NCI, NIH. PANC-1, A549, A549-DDP, BEL7404 and BEL7404-CP20 were grown in DMEM (Wisent, Canada) with 10% fetal bovine serum (FBS, Wisent, Canada) and 1% penicillin/streptomycin (Gibico, Invitrogen). SW480 was grown in L-15 (Wisent, Canada) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. PC-3 was grown in F12K (Wisent, Canada) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell lines were cultured at 37 ℃ in 5% CO_2. All cisplatin-resistant cell lines were grown in medium containing 5 μg/ml cisplatin.

***In vitro* anticancer activity assay**

The cytotoxicity of compounds to different cell lines were examined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells (4×10^3/well) were seeded in 96-well plates and cultured overnight. Then cells were incubated with cisplatin, flurbiprofen, platin-FP and cisplatin/FP (physical mixture of cisplatin and flurbiprofen, mole ratio 1:2) at a serial of concentrations ranging from 0.08 μM to 50 μM for 48 h. After that, cells were treated with 100 μl medium containing MTT (0.5 mg/ml) for 4 h, followed by replacing medium with 100 μl DMSO. The absorbance at 570 nm was measured using a microplate reader (Tecan, USA).

Cell apoptosis assay

A FITC Annexin V apoptosis detection kit I (BD Biosciences, USA) was used to test cell apoptosis. Cells (2×10^5/well) were seeded in 6-well plates and cultured overnight. Then cells were incubated with cisplatin (SW480: 5 μM; BEL7404 and BEL7404-CP20: 10 μM), flurbiprofen (SW480: 10 μM; BEL7404 and BEL7404-CP20: 20 μM), platin-FP (SW480: 5 μM; BEL7404 and BEL7404-CP20: 10 μM) and cisplatin/FP (SW480: 5 μM cisplatin/10 μM flurbiprofen; BEL7404 and BEL7404-CP20: 10 μM cisplatin/20 μM flurbiprofen) for 24 h under standard conditions. Cells without treating were used as controls. Next, the cells were harvested, washed, resuspended with 1×Annexin V binding buffer and stained by Annexin V-FITC and propidium iodide (PI). After staining, samples were analyzed using flow cytometry (Attune® NxT, ThermoFisher Scientific).

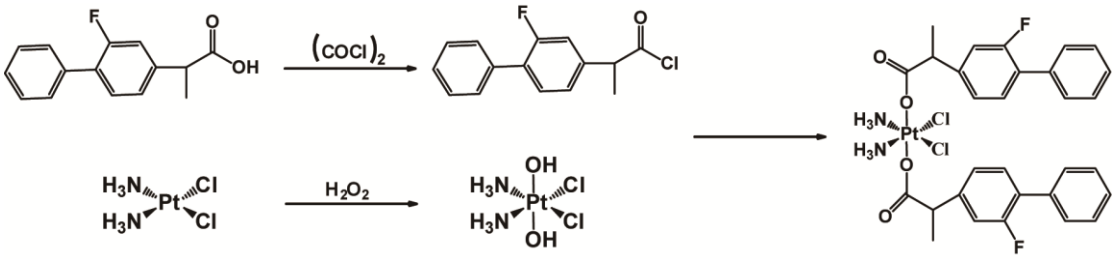
Cellular uptake and DNA platination

Cells were seeded in 100 mm^2 dishes and cultured under standard conditions. When cells were at about 90% confluence, the medium was replaced with fresh medium containing platin-FP (10 μM), cisplatin(10 μM) or cisplatin/FP (10 μM cisplatin/20 μM flurbiprofen). After 6 h, the medium was removed and the cells were washed with PBS solution three times. Then the cells were harvested, centrifuged and suspended in 6 ml PBS solution. The suspension was divided into two parts equally: one part was digested directly after the cell numbers were counted for analyzing the Pt concentration in the whole cell; another part was used to extract DNA for analyzing the DNA platination. DNA was extracted using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich), quantified by UV-visible spectroscopy (ES-2, Malcom, Japan) and digested by aqua regia. After digestion, the platinum content was determined by ICP-MS. Each experiment was repeated three times. The statistical significance was determined with the SPSS software. $P < 0.05$ was considered statistically significant.

Physicochemical properties of platin-FP nanoparticles formed by platin-FP

The platin-FP nanoparticles were prepared by dropwise adding 50 μl platin-FP/DMSO

solution into 10 mL water under stirring. The size distribution of the platin-FP nanoparticles was determined by dynamic light scattering (DLS) (Zetasizer 5000, Malvern Instrument Ltd., Worcestershire, UK). The morphology of the platin-FP nanoparticles was obtained using a transmission electron microscope (TEM) (HT7700, Hitachi, Japan), a scanning electron microscope (SEM) (SU8220, Hitachi, Japan) and a atomic force microscope (AFM) (Dimension 3100, Veeco, USA).



Scheme S1 Synthesis route of platin-FP.

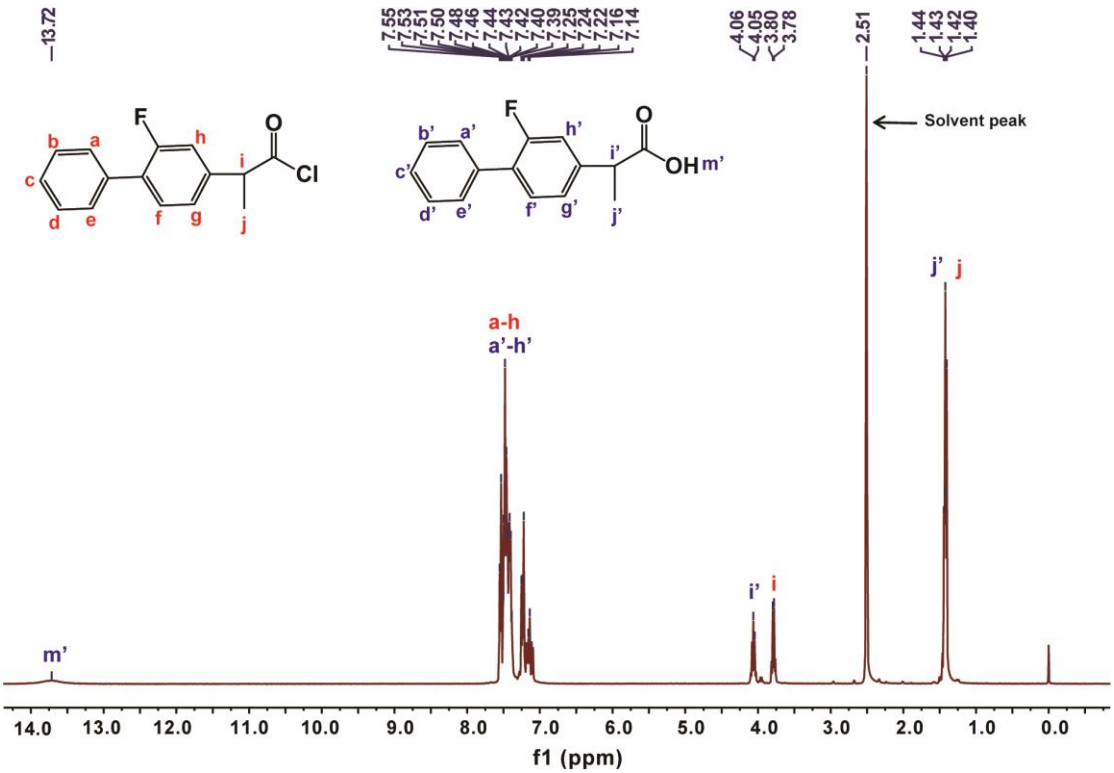


Figure S1 ^{1}H NMR spectrum of flurbiprofen chloride in DMSO-d^{6}, 400MHz. The peaks of a-j come from flurbiprofen chloride. Peaks of a'-j' and m' come from flurbiprofen, which is the hydrolysis product of flurbiprofen chloride with residual H_2O in DMSO-d^{6}.

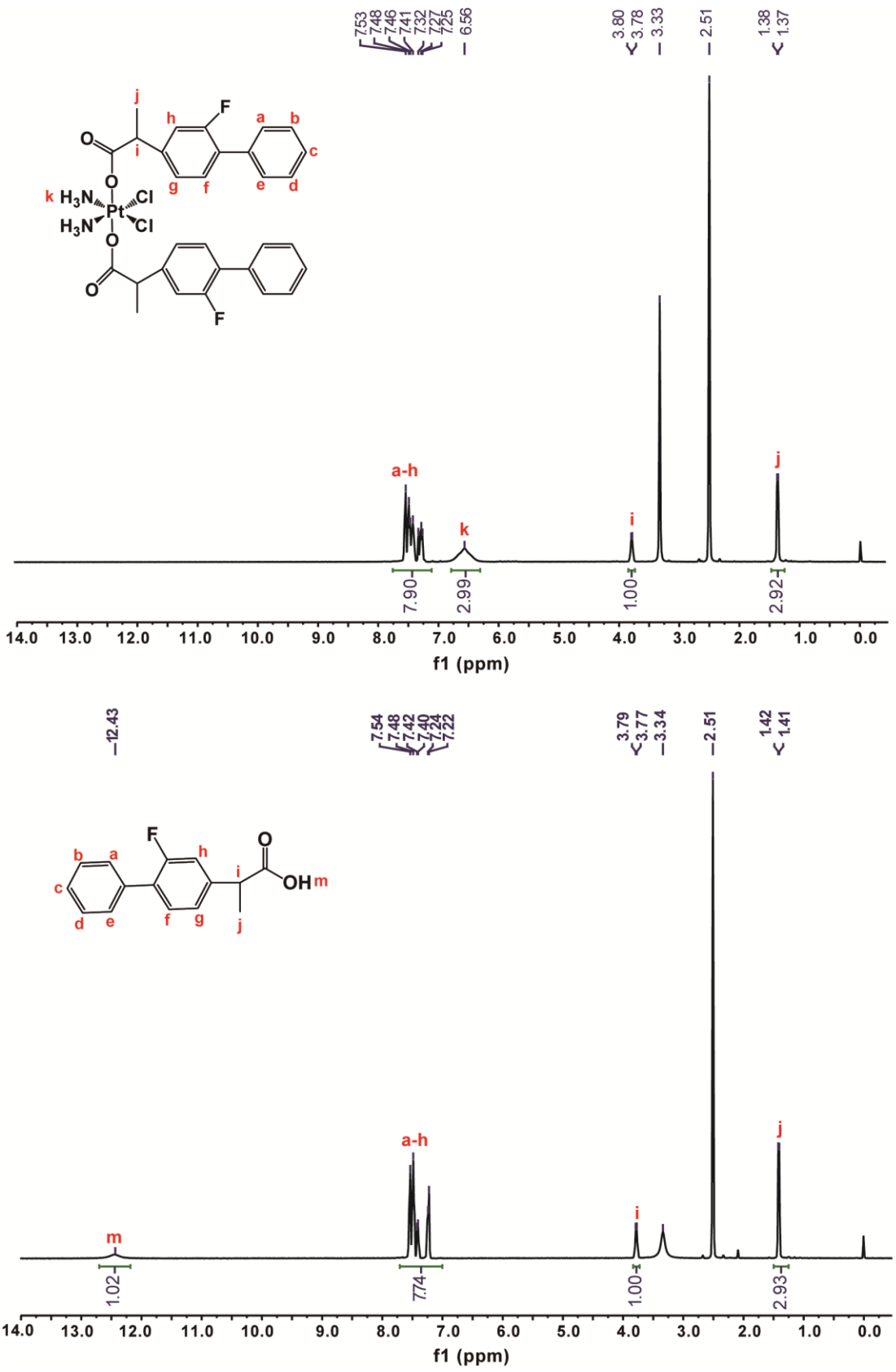


Figure S2 ^{1}H NMR spectra of platin-FP (top) and flurbiprofen (bottom) in DMSO-d^{6}, 400MHz.

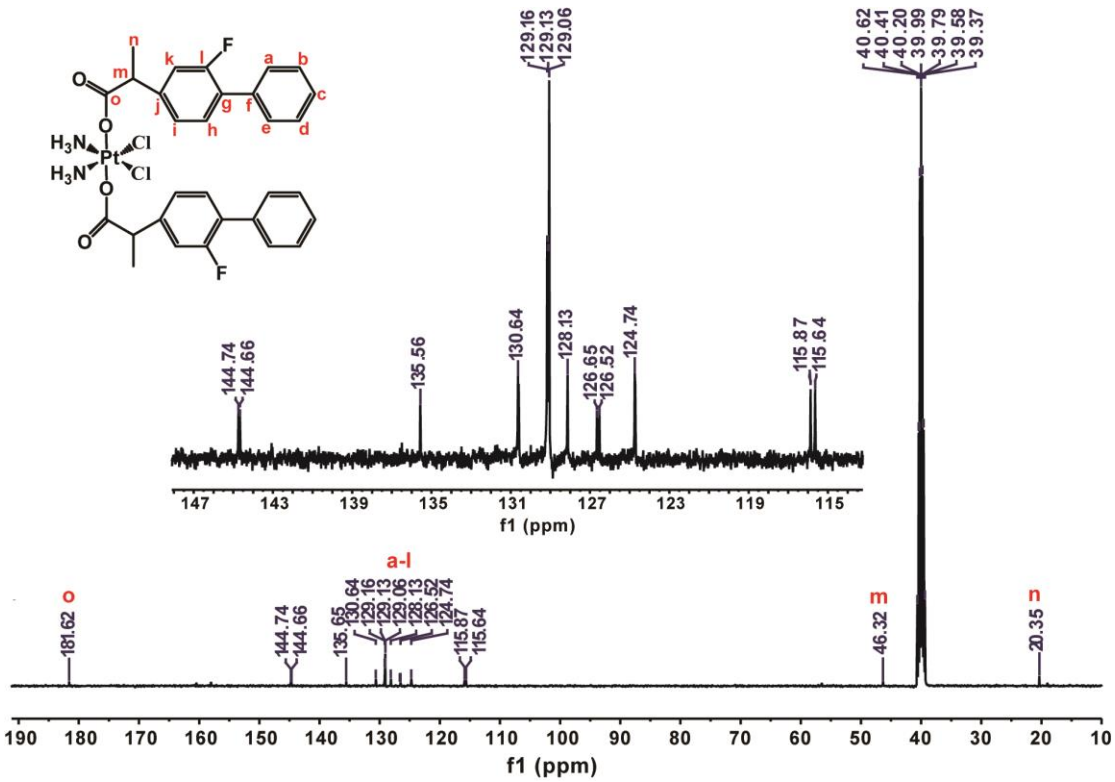


Figure. S3 ^{13}C NMR spectrum of platin-FP in DMSO-d^6, 400MHz.

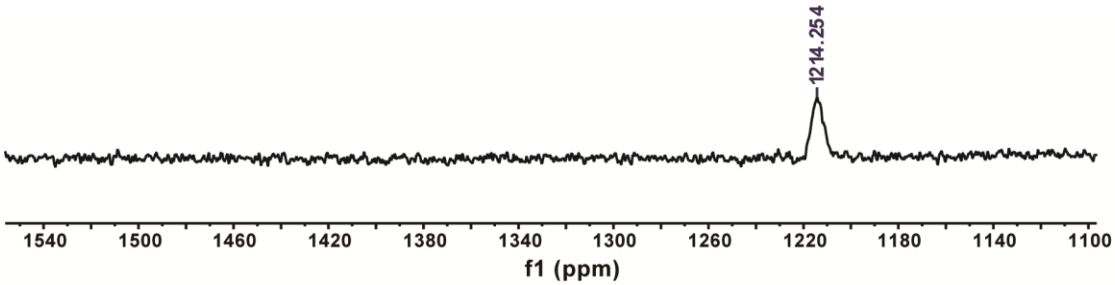


Figure S4 ^{195}Pt NMR spectrum of platin-FP in DMSO-d^6, 600 MHz.

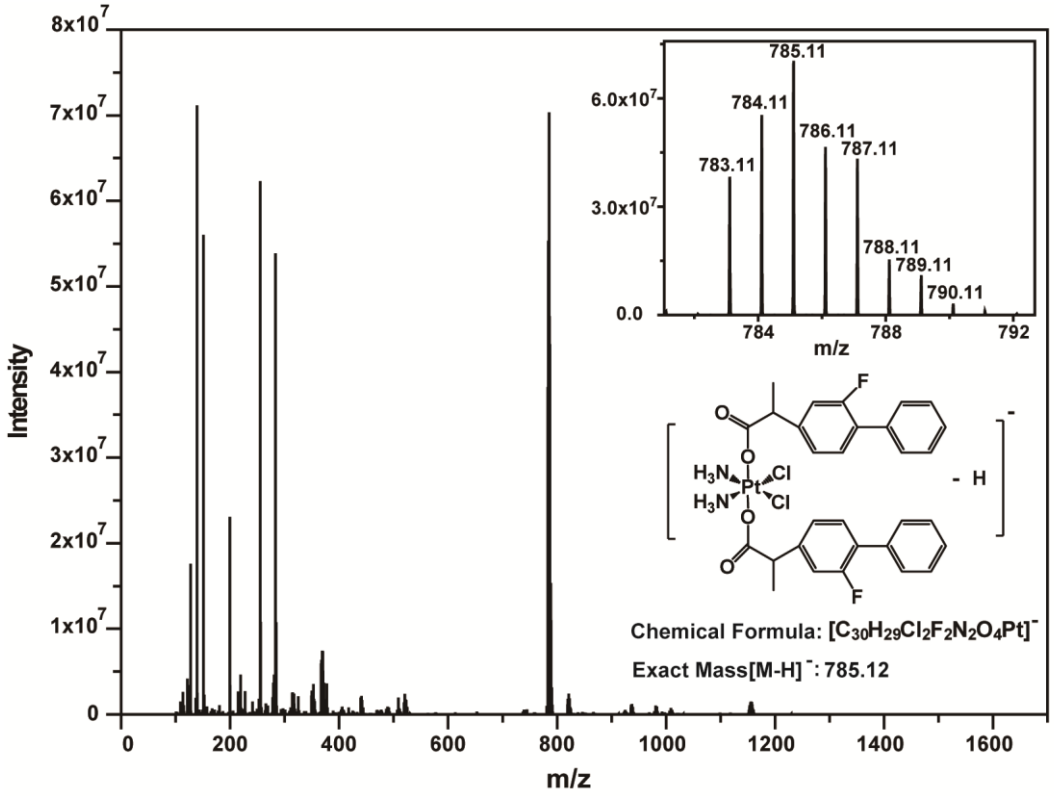


Figure S5 Negative ion ESI-MS spectrum of platin-FP.

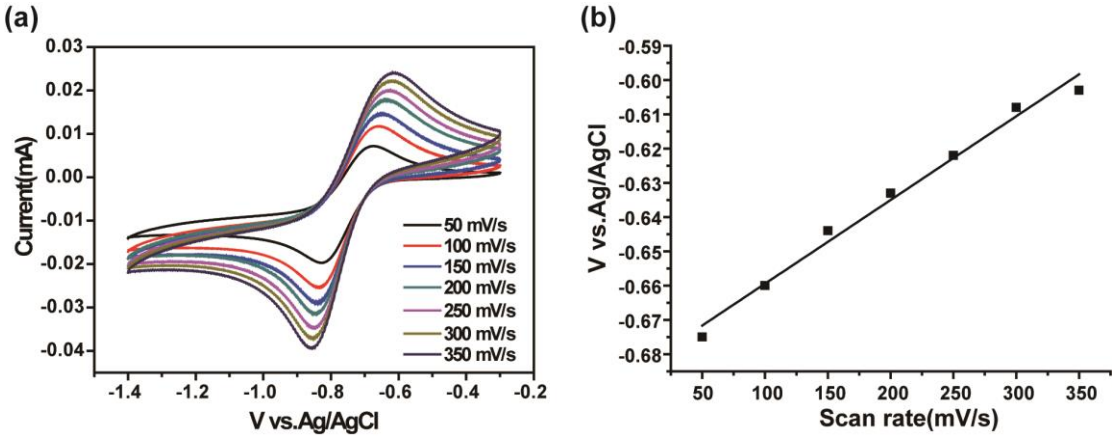


Figure S6 (a) Redox activity of platin-FP in DMSO-H_2O (3:1)-0.1 M $(C_4H_9)_4NClO_4$ with varied scan rates. (b) Plot of reduction peak potential maxima of (a) as a function of scan rate.

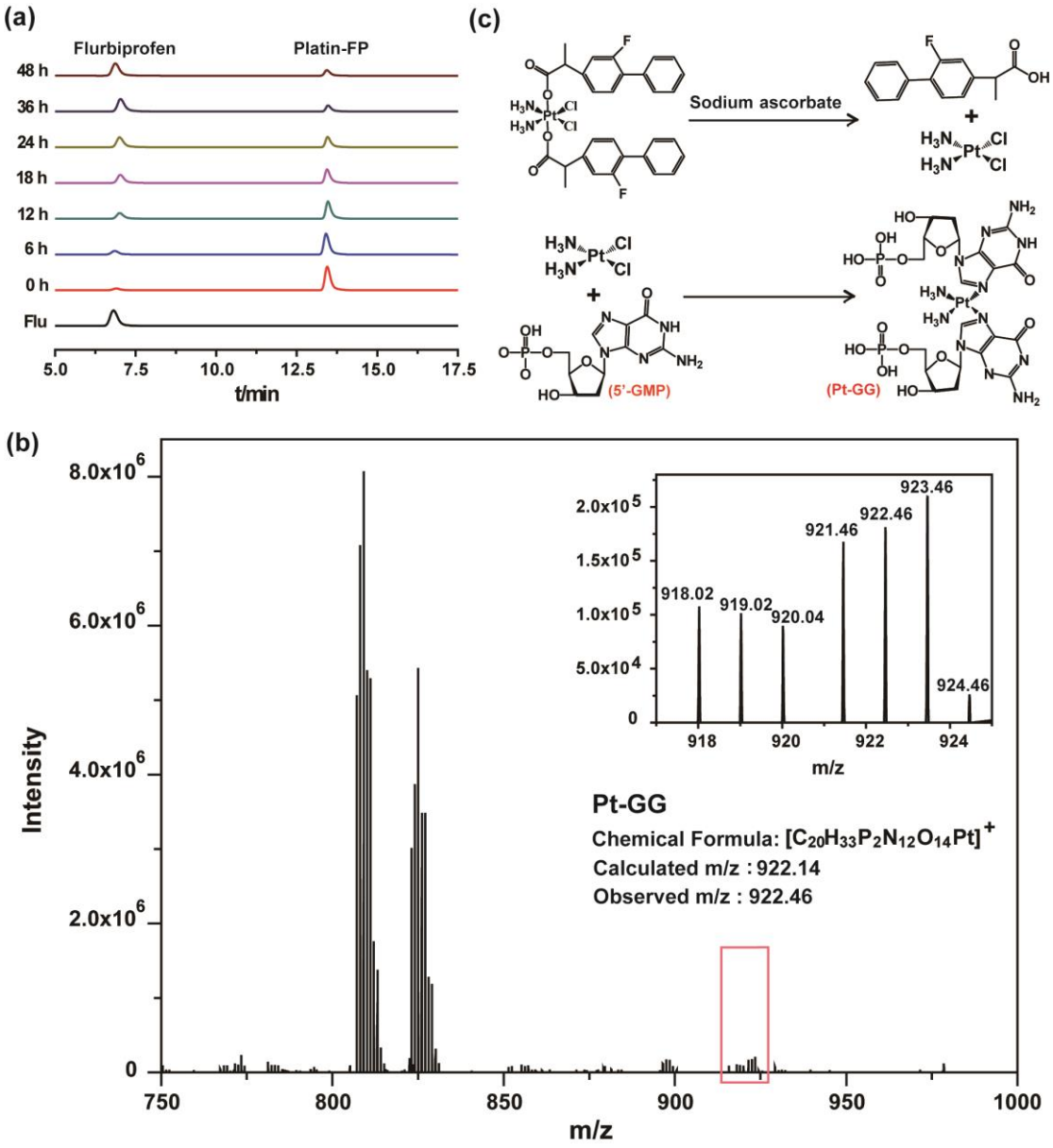


Figure S7 The reduction of platin-FP and reaction with 5'-GMP. (a) The reduction of platin-FP by ascorbic acid monitored using HPLC. (b) Formation of Pt-GG analyzed by ESI-MS. Calculated m/z = 922.14, observed m/z = 922.46. (c) The scheme of reduction of platin-FP and subsequent reaction with 5'-GMP.

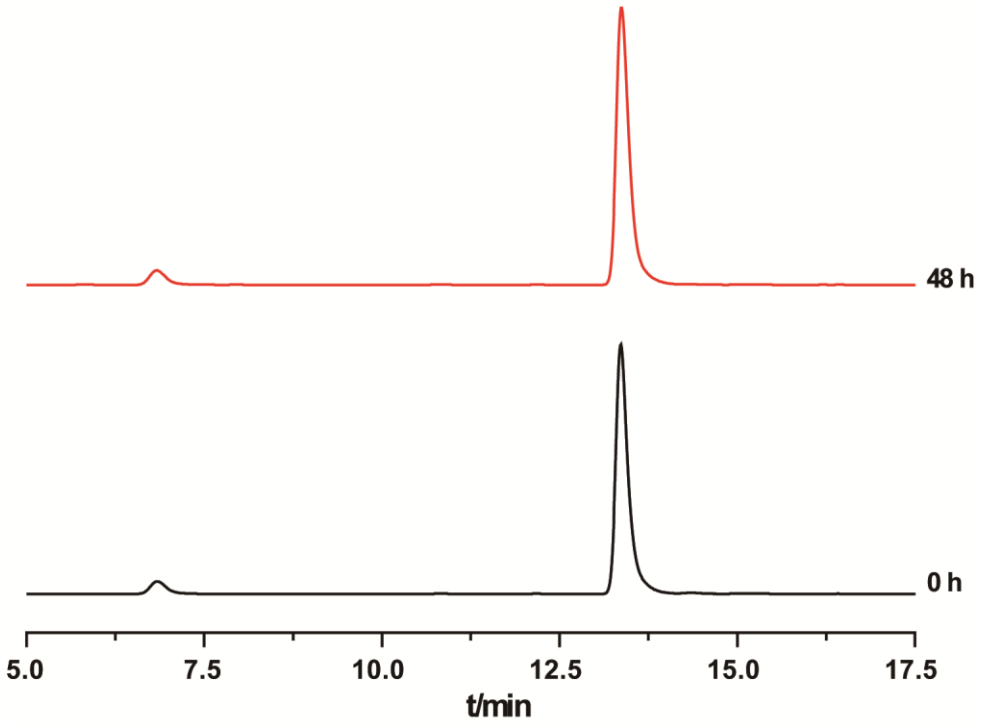


Figure S8 Stability of platin-FP without ascorbic acid at 37 ℃ was analyzed by HPLC.

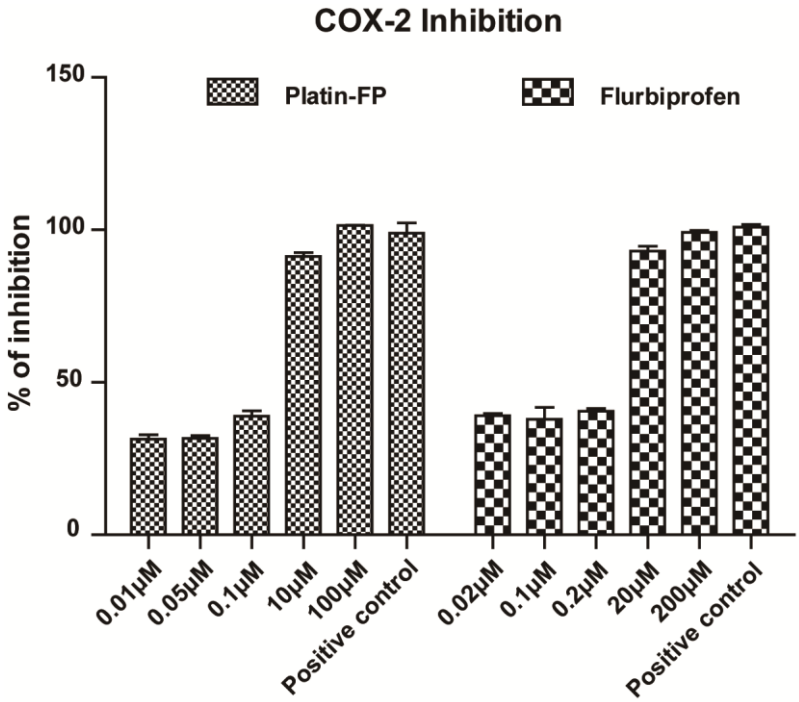


Figure S9 COX-2 inhibitory property of platin-FP at different concentrations

comparing with flurbiprofen.

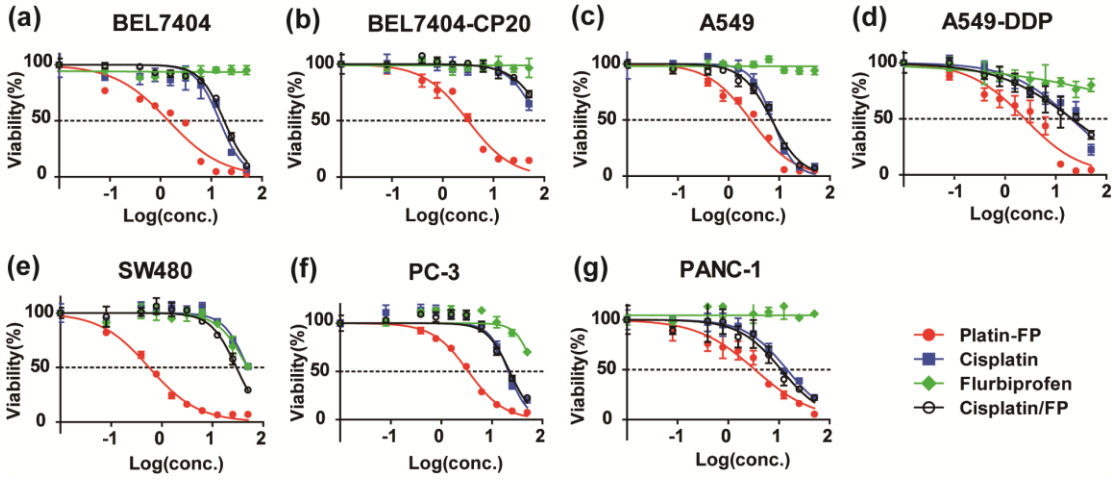


Figure S10 Cytotoxicity of platin-FP, cisplatin, flurbiprofen and cisplatin/FP (the mixture of cisplatin and flurbiprofen, mole ratio is 1:2) in different cancer cell lines.

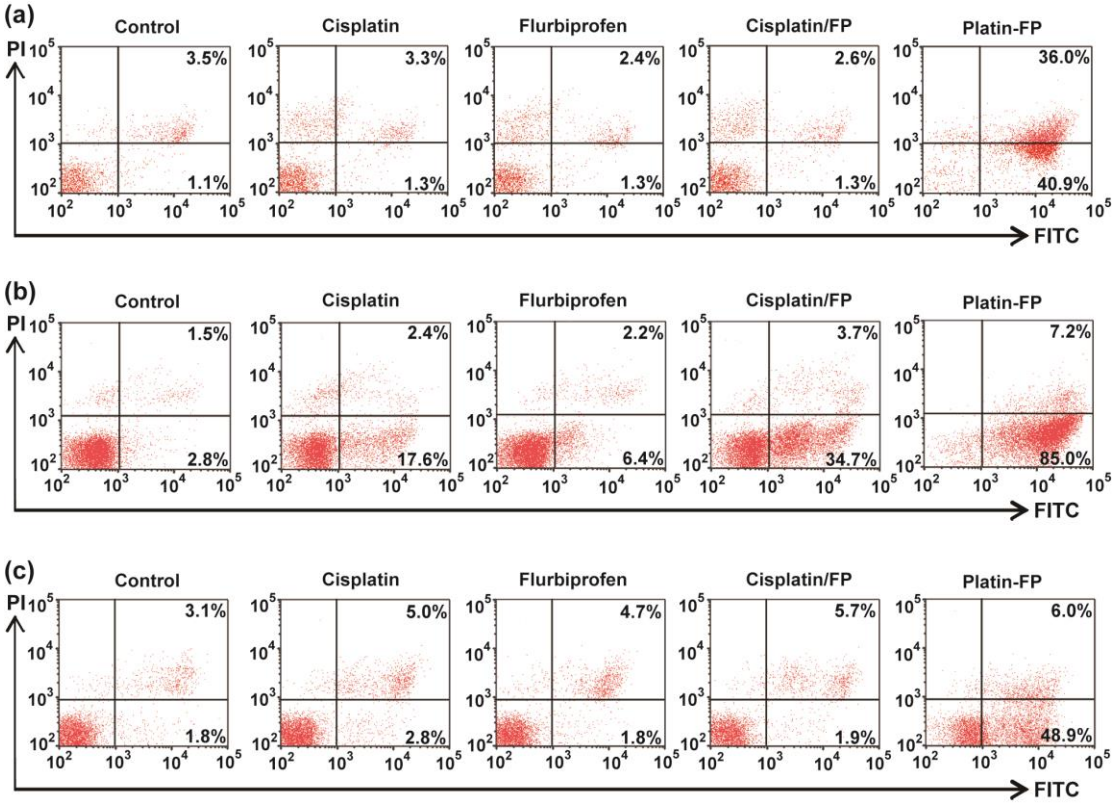


Figure S11 Quantification of cell apoptosis induced by platin-FP, cisplatin, flurbiprofen and cisplatin/FP (the mixture of cisplatin and flurbiprofen, mole ratio is 1:2) using annexin V-FITC/PI staining of different cancer cell lines. (a) SW480. (b) BEL7404. (c) BEL7404-CP20.

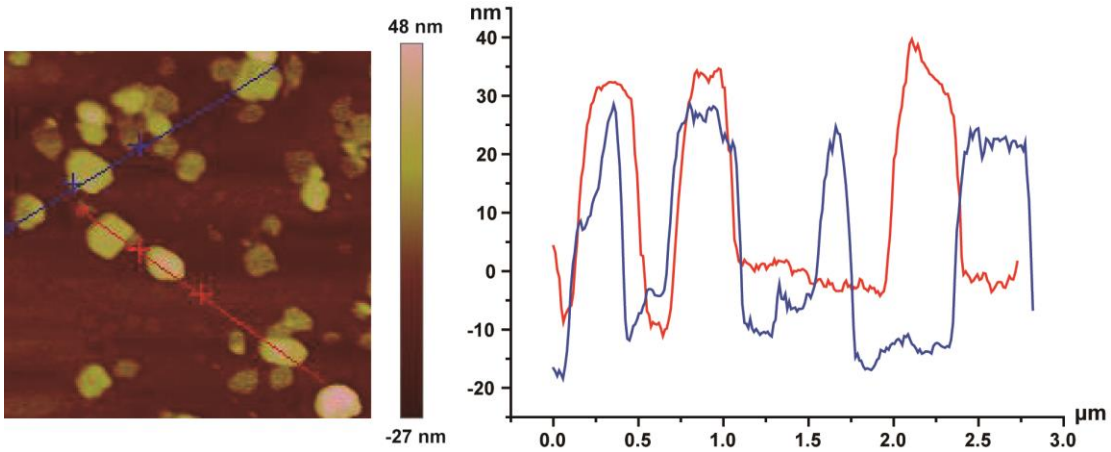


Figure S12 Characterization of nanostructure of platin-FP by AFM.