Electronic Supporting Information

for

The Ligation of Aspirin to Cisplatin Demonstrates Significantly Synergistic Effect to Tumor cells

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 (1). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT), ascorbic acid (AsA), glutathione (GSH), Herring sperm DNA, Hydrogen hydroxide (H_2O_2) (30% in H_2O), were purchased from Sangon Biotech (Shanghai) Co., Ltd. 5'-GMP was purchased from Shanghai Sanjie BioTechnology Co., Ltd. (purity ≥ 98.0%). 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 MTT assays was prepared by dissolving 20 g sodium dodecyl sulfate (SDS) in 50 mL ultrapure water supplemented with 50 mL N, N-dimethylformamide, pH 4.7.

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 cisplatin-resistant lung cancer cell line A549R was purchased from Shanghai Fumengjiyin biotechnology (FMGbio) Co., Ltd.). The cells were maintained in either DMEM (for HeLa, MCF-7 HepG2 cells) or RPMI1640 (for A549 and A549R cells) medium containing 10% fetal bovine serums in a humidified atmosphere containing 5% CO_2 at 37 °C. A549R cells were maintained with 2

μg/mL cisplatin. For MTT assay, the resistant cells were cultured in cisplatin free medium for 10 days before conducting cytotoxicity assay.

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.

Synthesis of asplatin. Oxoplatin was synthesized as described in the literature (1). H_2O_2 (3.5 mL 30% w/v, 0.03 mol) was added to the suspension of 100 mg cisplatin (0.33 mmol) in 5 mL water. The mixture was stirred for 1 h at 50ºC and then for another 12 h at room temperature. The solution was lyophilized. The resultant was washed with cold water, ethanol and ether, and dried again in vacuum.

For the preparation of asplatin, 204 mg acetylsalicylic anhydride (0.6 mmol) was added to 10 mL dimethylsulfoxide (DMSO) solution of oxoplatin (100 mg, 0.3 mmol). The mixture was stirred for 24 h at room temperature. The solution was lyophilized and the resultant was washed with acetone and diethyl ether to yield a pale yellow solid. The product was dried in vacuum and was characterized by HPLC, HNMR, ESI-MS.

In Vitro Cellular Cytotoxicity Assays. Cells were seeded in 96-well plates at 3000 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 48 h, and then the medium in each well 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 the 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), which were based on three parallel experiments.

HPLC Studies. HPLC analyses were performed as on an Agilent 1200 system equipped with a Kromasil C18 column (250×4.6 mm, 5 μm). HPLC profiles were recorded by UV detector at 280 nm.

Reactions of asplatin with DNA: Herring sperm DNA was dissolved in 10 mM phosphate buffer, pH 7.4 containing 10 mM $NaClO_4$. The DNA concentration was determined by UV-vis spectra at 260 nm with extinction coefficient 6600 M^{-1}. The mixture of DNA with platinum compounds was incubated at 37°C in the dark. The fluorescence was measured in 0.4 M NaCl to avoid the second fixation site of EtBr to DNA (2). EtBr (0.04 mg/mL) was added to the 0.01 mg/mL DNA solution before the fluorescence measurement. 1 mM AsA or 1.6 mM GSH was added for the reduction of asplatin. Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrometer using a 1 cm path length cuvette under following conditions: scan speed 2000 nm·min^{-1}; excitation slit width was 5 nm and emission slit width was 10 nm. The excitation and emission wavelength was 530 nm and 592 nm, respectively.

The reactions of oligomeric DNA were performed on the palindromic ds-DNA dodecamer $d(AATTGGCCAATT)_2$ in 10 mM phosphate buffer, pH 7.4 containing 10 mM $NaClO_4$. The mixture of DNA with platinum compounds was incubated at 37°C in the dark. The product was monitored using HPLC. HPLC analyses were performed as on an Agilent 1200 system equipped with a Kromasil C18 column (250×4.6 mm, 5 μm). The linear gradient was used from 10-20% elution B in 0~15min. (eluents: A: 50 mM ammonium acetate in H_2O; B: acetonitrile). HPLC profiles were recorded by UV detector at 260 nm.

Cellular Platinum Uptake and DNA Platination. The cellular uptake of cisplatin, oxoplatin and asplatin was measured on HeLa cells. HeLa cells were seeded in 6-well plates overnight and then incubated with 100 μM drugs at 37 °C in standard culture conditions for 3 h. Then the cells were washed with PBS buffer (pH 7.4) for three times, and harvested by trypsinization. The harvested cells were concentrated and digested by nitric acid for the ICP-MS. The cell numbers were counted before the digested. For the measurement of Pt concentration in cellular DNA in HeLa cells, DNA was isolated by using Genomic DNA Mini Preparation Kit (Beyotime, China), and Pt content in DNA was analysised using ICP-MS.

Apoptosis analysis with annexinV/PI assay. HeLa cells cultured in 12-well plates were treated with drugs for 30 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.

In vivo antitumor assay. The HepG2 single-cell suspension in PBS (5×10^6 per mouse) was injected subcutaneously into the buttock of the mouse. When the tumor grew to a size of 80-150 mm^3 at 12 days after cell implantation, the 40 mice were randomly divided into five treatment groups (PBS, cisplatin 0.5 mg Pt/kg, cisplatin 2 mg Pt/kg, asplatin 0.5 mg Pt/kg and asplatin 2 mg Pt/kg), with all drugs administered intravenously via tail vein. The drugs were given 5 times at 3-day intervals. Tumor growth was monitored by measuring the perpendicular diameter of the tumor using calipers every two days and calculated according to the formula: tumor volume (mm^3) = 0.5 × length × $width^2$. Five days after the last treatment, animals were sacrificed and kidneys and tumors were excised for immunohistochemistry analysis.

Statistical Analysis. The statistical significance of treatment was assessed using the Prism software (GraphPad); The statistical differences were determined by ANOVA followed by Newman Keuls Post Hoctest or Student's t test. Values $p < 0.05$ indicate significant differences.

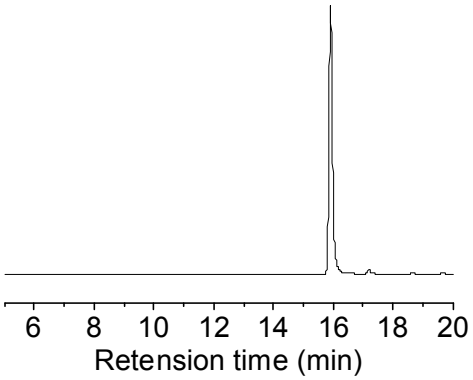

Figure S1. HPLC characterization of the purity of asplatin.

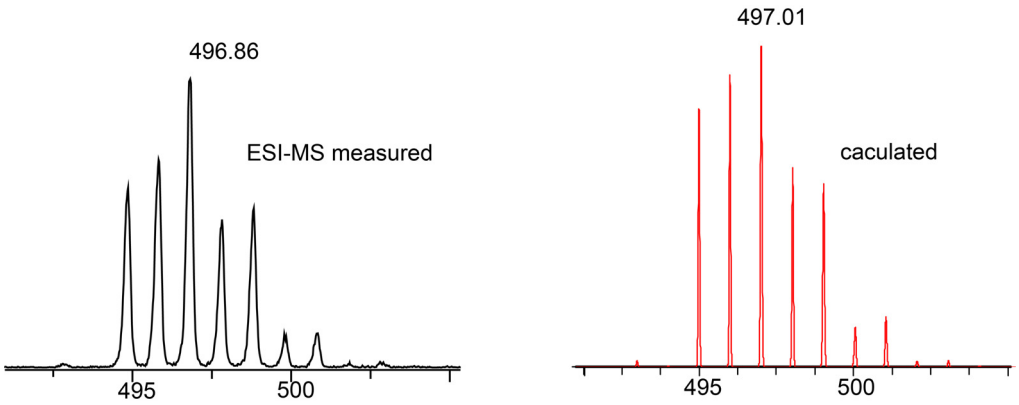

Figure S2. ESI-MS of asplatin measured in the positive mode. Composition: *c,c,t*-$[Pt(NH_3)_2Cl_2(C_9H_7O_4)OH+H]^+$. The right portion shows the theoretically simulated spectrum of asplatin. The measured m/z is 496.86 and the calculated m/z is 497.01.

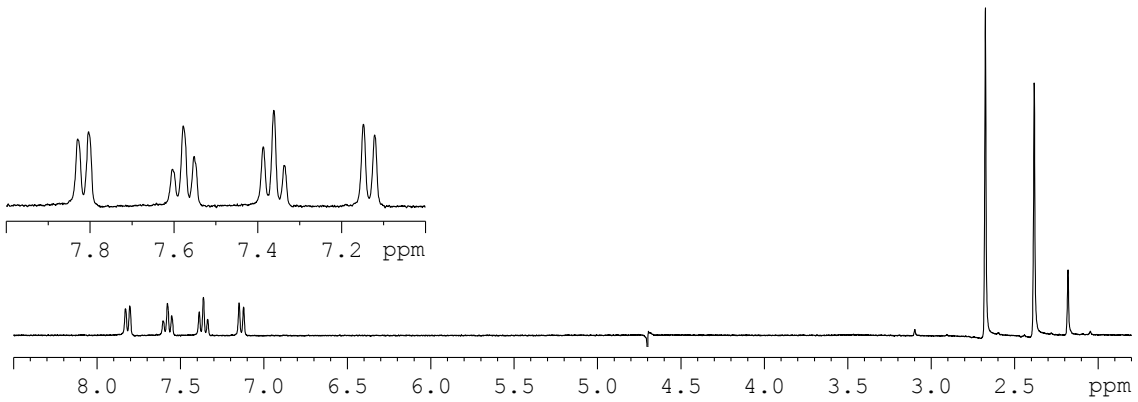

Figure S3. ^{1}H NMR spectrum of asplatin, *c,c,t*-$[Pt(NH_3)_2Cl_2(C_9H_7O_4)OH]$ in D_2O. The spectrum was recorded using presaturation pulse sequence to suppress the residue HDO. The inset shows the expansion of the aromatic proton signals.

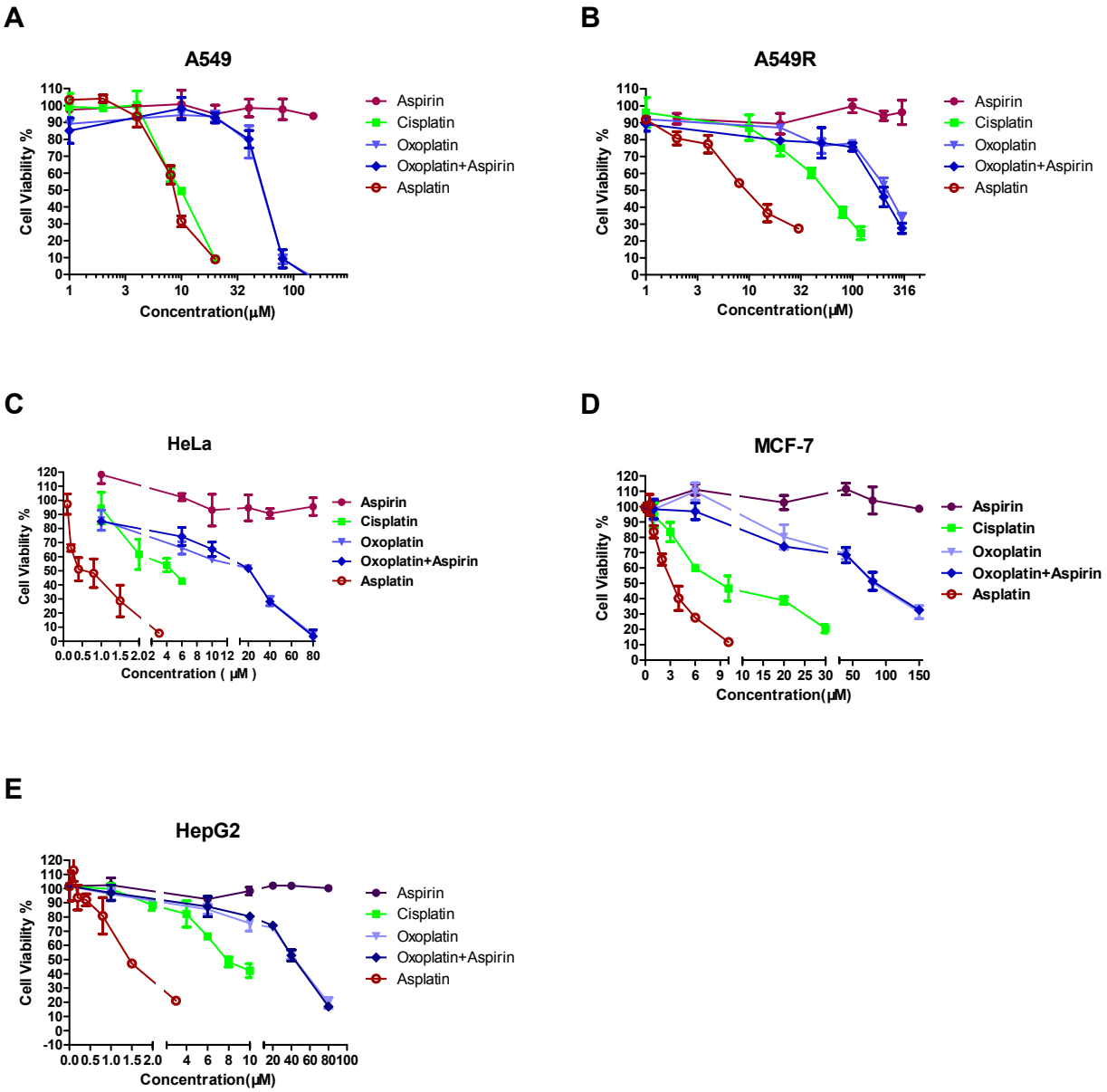

Figure S4. Cytotoxicity assay of aspirin, cisplatin, oxoplatin, oxoplatin with equimolar aspirin, and asplatin. (A) A549 cells, (B) A549R cells, (C) HeLa cells, (D) MCF-7 cells, (E) HepG2 cells.

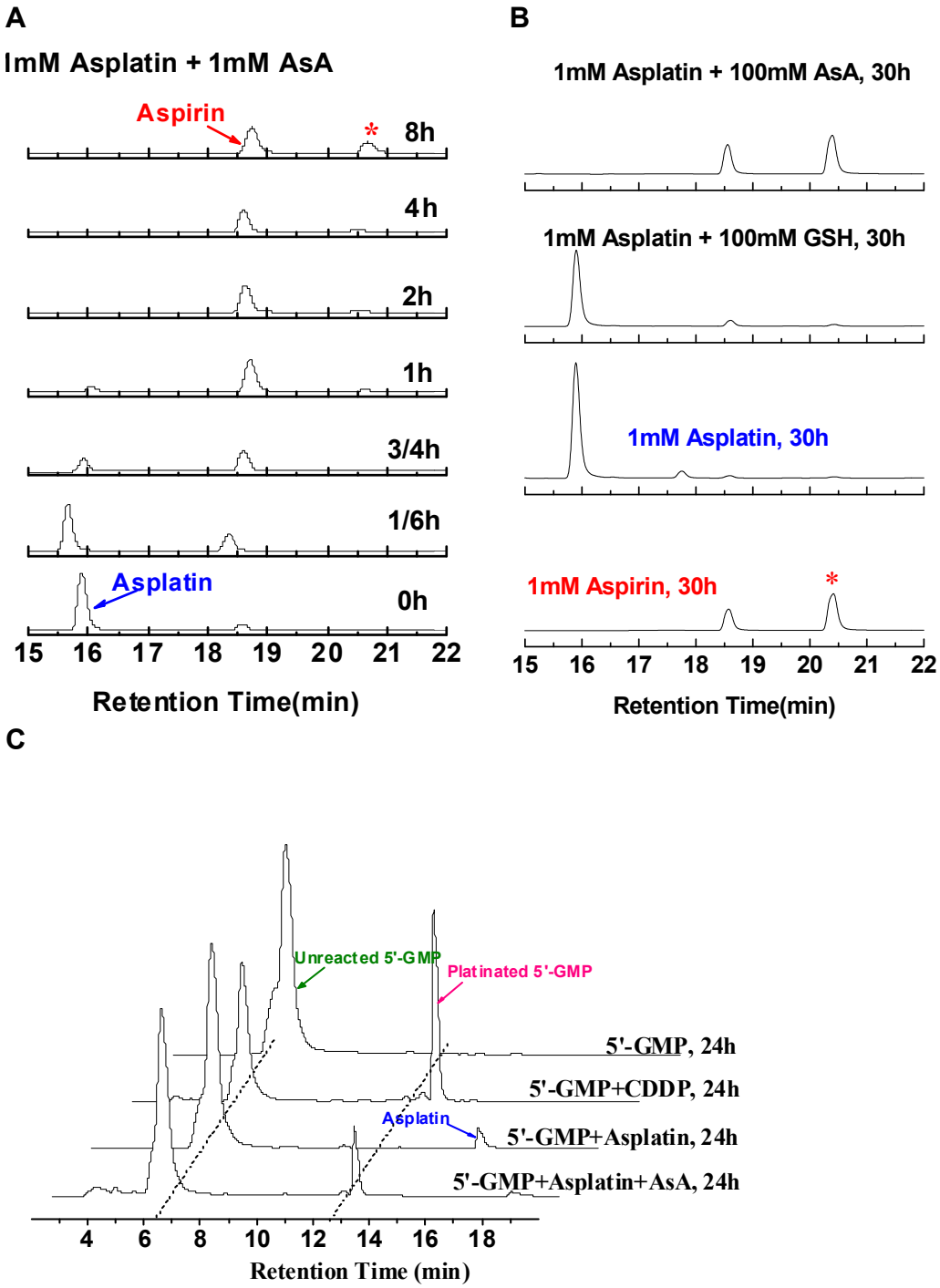

Figure S5. The reduction of aspirin and the reaction with GMP. (A) The reduction of asplatin with AsA at 37 °C. The asterisk denotes the hydrolysis of aspirin. (B) The comparison of reactions of asplatin with GSH and AsA. (C) HPLC profiles of 1 mM 5'-GMP in the reaction of 1 mM platinum complexes for 24 hours. 5 mM AsA was added for the reduction of asplatin. The linear gradient was used from 10-100% elution B in 0~30 min. (eluents: A: 0.1% CF_3COOH in H_2O; B: CH_3OH).

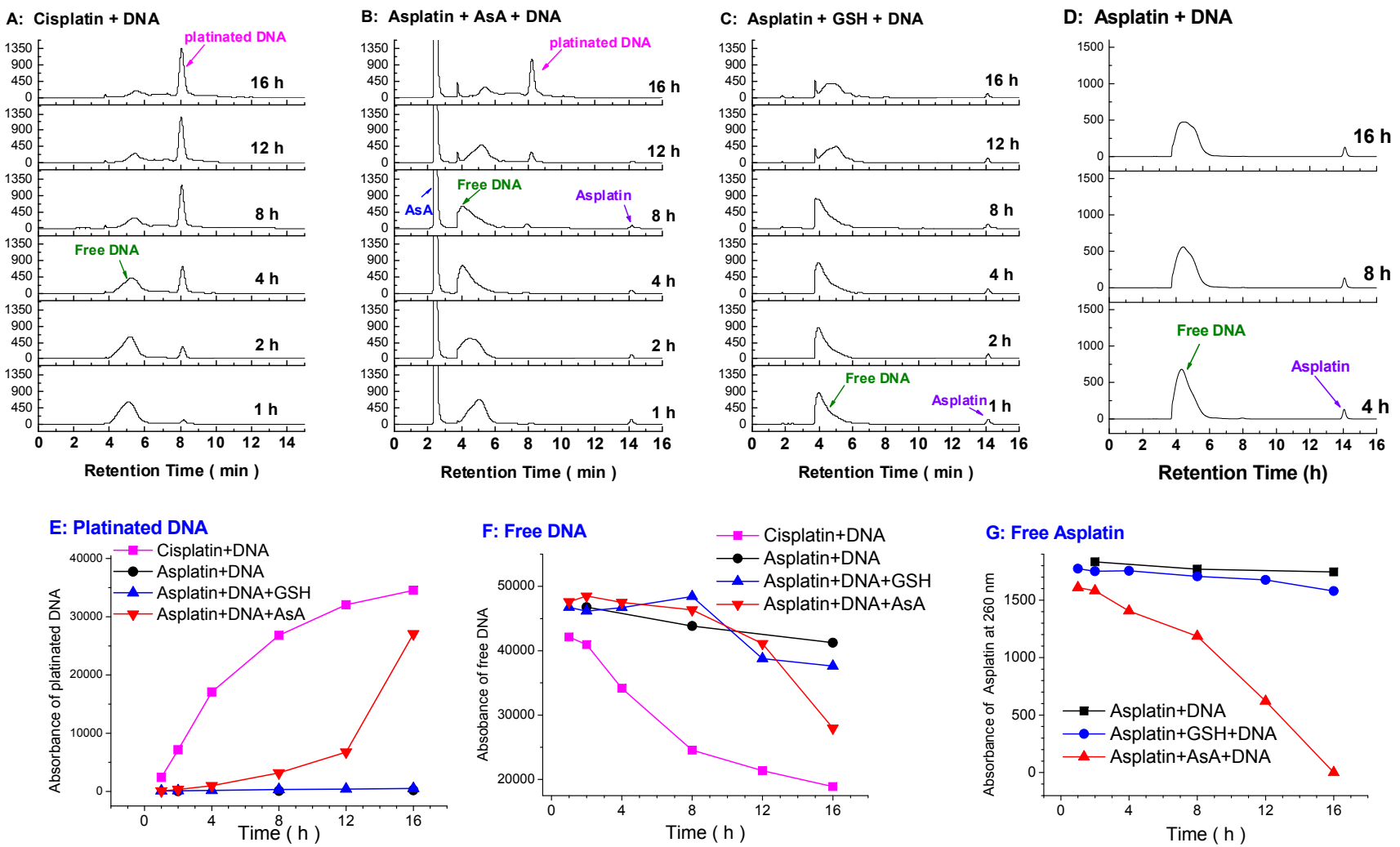

Figure S6. The reaction of aspirin with ds-DNA oligomer. (A) - (D) HPLC profiles of 40 μM ds-DNA (sequence: d[AATTGGCCAATT]$_2$) in the reaction of 200 μM platinum complexes at 37 °C. 5 mM GSH or AsA was added for the reduction of asplatin. The linear gradient was used from 10-20% elution B in 0~15min. (eluents: A: 50 mM ammonium acetate in H_2O; B: acetonitrile) (E) - (G): The integral of HPLC peaks. (E) platinated ds-DNA; (F) free DNA; (G) free asplatin.