Supporting Information for

Heterodinuclear Pt(IV)–Ru(II) anticancer prodrugs to combat both drug resistance and tumor metastasis

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Experimental section

1. Materials

All reactions were carried out under argon atmospheric conditions. Agents and solvents were used as received without additional drying or purification unless otherwise indicated.

2. Methods and measurements

2.1 General measurements.

^{1}H, ^{13}C, and ^{195}Pt NMR spectra were measured by Bruker Ultrashield 300 MHz, 400 MHz, or 600 MHz NMR spectrometer at ambient temperature. All NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced as described below. ^{1}H and ^{13}C NMR spectra were referenced internally to residual solvent peaks using deuterated chloroform ($CDCl_3$), deuterated dimethyl sulfoxide (DMSO-d_6), or deuterated dimethyl formamide (DMF-d_7) as the solvent. ^{195}Pt NMR spectrum was referenced externally using standards of K_2PtCl_4 in D_2O (δ = -1628 ppm). ESI-MS was carried out on an Agilent API-2000 mass spectrometer. Methanol was used as solvent. IR spectra were tested on a Perkin Elmer Spectrum 2000 by using OMNIC software at r.t.. Samples were prepared as KBr disks. Elemental analysis was performed by using a Vario Micro elemental analyzer. Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Optima 2100DV, PerkinElmer, USA) was applied to determine platinum and ruthenium levels. Cyclic voltammograms were obtained at room temperature using an electrochemical analyzer system CHI 660C. Ion chromatography was measured by using a Dionex ICS-1600 system.

2.2 X-ray crystallography

Orange crystals were obtained by diffusing diethyl ether (Et_2O) into a saturated solution of PPA-Ru(II) **5** in dichloromethane (DCM). When diffusing a mixture of DCM/Et_2O into a

solution of PPA-Pt(IV) in dimethylsulfoxide (DMSO), light yellow crystals were harvested. Crystals with suitable size coated with paratone-N and mounted on a nylon cryoloop were used for X-ray diffraction analysis. X-ray diffraction data were collected using an ω-scan mode at 193 K on an Oxford Diffraction Gemini S Ultra 4-circle kappa diffractometer with a 92 mm diagonal Sapphire CCD detector using either mirror-monochromatized Cu-*K*α radiation (λ = 1.5418 Å) or graphite-monochromatized Mo-*K*α radiation (λ = 0.7107 Å). The data were processed and absorption correction was done by a multi-scan method using CrysAlis. Crystal data and experimental details are listed in Tables S3 and S5. Selected bond angles and bond lengths are given in Tables S2 and S4. Structures of PPA-Ru(II) **5** and PPA-Pt(IV) were solved and refined using full-matrix least-squares based on F^2 with program SHELXS-97 and SHELXL-97 within Olex2. Ruthenium (in PPA-Ru(II) **5**), platinum (in PPA-Pt(IV)) and many non-hydrogen atoms were located according to direct method. The positions of other non-hydrogen atoms were found after successful refinement using program SHELXL-97. In the final stage of refinement, all non-hydrogen atoms were refined anisotropically. H atoms were generated by program SHELXL-97. The positions of H atoms were calculated based on riding model with thermal parameters equal to 1.2 times (1.5 times for H atoms in methyl groups) that of the associated C atoms and N atoms, and these are participated in the calculation of final *R*-indices.

2.3 Stability test

^{1}H NMR spectra. Solutions of PPA-Pt(IV) (1.3 mM), ruthplatin **1** (4 mM), and PPA-Ru(II) **5** (4 mM) were prepared by dissolving the powders of corresponding complexes into D_2O just before the experiments. The spectra were recorded at different time points, and residue organic solvent from synthesis step, such as DMF, was used as an internal reference. ^{1}H NMR spectra in D_2O were acquired on a Bruker Ultrashield 400 MHz or 600 MHz NMR spectrometer.

3. Synthesis and characterizations

All the complexes were well characterized by ^{1}H, ^{13}C, and ^{195}Pt NMR spectroscopy (when Pt is included) as well as ESI-MS and CHN elemental analysis (Figure S1–S16, Table S1). The CHN analysis data of PPA-Pt(IV), PPA-Ru(II) **5**–**8**, ruthplatin **1**–**4**, CymRu(II)(O^O)H_2O and silver oxalate were showed in Table S1.

3.1 Synthesis route of PPA-Pt(IV)

Synthesis of *c*,*c*,*t*-[Pt(NH$_3$)$_2$Cl$_2$(OH)$_2$]: *c*,*c*,*t*-[Pt(NH$_3$)$_2$Cl$_2$(OH)$_2$] was synthesized as previously described[1].

Synthesis of PPA NHS ester: 50 mL acetonitrile was added to a mixture of 3-pyridinepropionic acid (PPA) (1.0 g, 6.62 mmol), *N*-hydroxysuccinimide (NHS, 0.914 g, 7.28 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 1.52 g, 7.28 mmol) to get a suspension. After 12 h stirring at r.t., a colorless solution was obtained. Acetonitrile was removed by rotary evaporation to yield a white-yellow raw product. The product was dissolved

in DCM and washed with water. Organic layer was collected, and $MgSO_4$ was added to remove water. Finally, a white powder was gained by removing the solvent. Yield: 1.0 g, 61%. ^{1}H NMR (300 MHz, DMSO-d_6) δ_{ppm} 8.53 (d, J = 2.0 Hz, 1H, py), 8.43 (dd, J = 4.7, 1.3 Hz, 1H, py), 7.74 (d, J = 7.8 Hz, 1H, py), 7.33 (dd, J = 7.8, 4.8 Hz, 1H, py), 3.08 (t, J = 7.2 Hz, 2H, CH_2), 2.96 (dd, J = 18.5, 11.0 Hz, 2H, CH_2), 2.81 (s, 4H, $2CH_2$).

Synthesis of PPA-Pt(IV): A solution of PPA NHS ester (446.5 mg, 1.80 mmol) in DMSO (extra dry, 20 mL) was added dropwise to a suspension of *cis,cis,trans*-$[Pt(NH_3)_2Cl_2(OH)_2]$ (400 mg, 1.20 mmol) in DMSO (extra dry, 30 mL) with vigorously stirring. The mixture was stirred at r.t. for 72 h, then an excess amount of Et_2O was added to the yellow solution to get a white precipitate. The light-yellow precipitate was washed with DCM and dried. White powder. Yield: 400 mg, 71.4%. ^{1}H NMR (400 MHz, DMF-d_7) δ_{ppm} 8.49 (d, J = 1.9 Hz, 1H, py), 8.41 (dd, J = 4.7, 1.5 Hz, 1H, py), 7.71 (d, J = 7.8 Hz, 1H, py), 7.29 (dd, J = 7.8, 4.8 Hz, 1H, py), 6.57 – 6.07 (m, 6H, $2NH_3$), 2.84 (t, J = 7.7 Hz, 2H, CH_2), 2.52 (t, J = 7.7 Hz, 2H, CH_2), 1.28 (s, 1H, OH). ^{13}C NMR (100 MHz, DMF-d_7) δ_{ppm} 181.6, 151.0, 148.3, 138.5, 136.9, 124.4, 41.5, 38.8. ^{195}Pt NMR (129 MHz, DMSO-d_6) δ_{ppm} 1050.8. MS (ESI^+) *m/z*: $[M+H]^+$ calculated for C8H16N3O3Cl2Pt1: 468.2, found: 468.0. IR (KBr, cm^{-1}): 3429 (s), 3216 (m), 3117 (w), 1634 (vs), 1356 (s), 581 (m).

3.2 Synthesis of [(arene)Ru(II)Cl(μ-Cl)$_2$]$_2$

The [(arene)Ru(II)Cl(μ-Cl)$_2$]$_2$ were synthesized according to the previous reports[2]. The main procedure is to reflux hydrated $RuCl_3 \cdot nH_2O$ and excess amount of corresponding diene (*α*-phellandrene, 1-methyl-1,4-cyclohexadiene, or 1,3-cyclohexadiene) in ethanol for 4–6 h, then red or brown-red precipitates were filtrated and washed with ethanol and Et_2O. Red or brown-red powder were obtained by drying.

[(η^6-*p*-cym)Ru(II)Cl(μ-Cl)$_2$]$_2$ ^{1}H NMR (400 MHz, DMF-d_7) δ_{ppm} 5.68 (d, J = 5.9 Hz, 2H), 5.45 (d, J = 5.9 Hz, 2H), 2.99 – 2.90 (m, 1H, CH), 2.19 (s, 3H, CH_3), 1.33 (d, J = 6.9 Hz, 6H, $(CH_3)_2$).

[(π-methyl-C$_6$H$_6$)Ru(II)Cl(μ-Cl)$_2$]$_2$ ^{1}H NMR (300 MHz, DMF-d_7) δ_{ppm} 5.81 (t, J = 5.7 Hz, 2H, arene), 5.60 (t, J = 5.5 Hz, 1H, arene), 5.52 (d, J = 5.8 Hz, 2H, arene), 2.20 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMF-d_7) δ_{ppm} 99.1, 85.9, 80.0, 76.8, 19.0.

[(π-C$_6$H$_6$)Ru(II)Cl(μ-Cl)$_2$]$_2$ ^{1}H NMR (400 MHz, DMSO-d_6) δ_{ppm} 5.98 (d, J = 0.6 Hz, 6H).

3.3 Synthesis of ruthpaltin 1–3

Synthesis of ruthplatin 1: [(η^6-*p*-cym)Ru(II)Cl(μ-Cl)$_2$]$_2$ (65.5 mg, 0.107 mmol) and PPA-Pt(IV) (100.0 mg, 0.214 mmol) were stirred in dimethyl formamide (DMF) (20 mL) at r.t. in dark for 12 h. Then Et_2O was added to the filtered solution to get a yellow precipitate. The final product was separated by centrifugation and washed by Et_2O to obtain a yellow powder. Yield: 110.0 mg, 66.5 %. ^{1}H NMR (400 MHz, DMF-d_7) δ_{ppm} 8.91 (s, 1H, py), 8.85 (d, J = 5.5 Hz, 1H, py), 7.88 (d, J = 8.0 Hz, 1H, py), 7.39 (dd, J = 7.6, 5.8 Hz, 1H, py), 6.34 (m, 6H, $2NH_3$), 5.65

(d, J = 6.0 Hz, 2H, arene), 5.47 (d, J = 5.9 Hz, 2H, arene), 2.95 – 2.89 (m, 1H, CH), 2.86 (t, J = 7.5 Hz, 2H, CH_2), 2.56 (t, J = 7.5 Hz, 2H, CH_2), 2.01 (s, 3H, CH_3), 1.31 (d, J = 6.9 Hz, 6H, $2CH_2$). ^{13}C NMR (100 MHz, DMF-d_7) δ_{ppm} 180.4, 155.3, 152.6, 138.4, 138.23, 124.0, 102.6, 97.4, 83.72, 82.0, 37.4, 30.9, 21.9. ^{195}Pt NMR (129 MHz, DMF-d_7) δ_{ppm} 996.0. MS (ESI$^+$) *m/z*: [M–Cl]$^+$ calculated for C18H29N3O3Cl3Ru1Pt1: 737.9, found: 738.0. IR (KBr, cm^{-1}): 3443 (s), 3226 (m), 3071 (w), 2960–2871 (w), 1634 (s), 1384 (s), 1319 (m), 586 (w).

Ruthplatin **2** was synthesized using the same method as ruthplatin **1**. Yellow powder. Yield: 100.0 mg, 57.5%. ^{1}H NMR (400 MHz, DMF-d_7) δ_{ppm} 8.95 (s, 1H, py), 8.87 (d, J = 5.2 Hz, 1H, py), 7.87 (d, J = 7.9 Hz, 1H, py), 7.36 (dd, J = 7.6, 5.8 Hz, 1H, py), 6.60 – 6.08 (m, 6H, $2NH_3$), 5.79 (t, J = 5.6 Hz, 2H, arene), 5.71 (t, J = 5.4 Hz, 1H, arene), 5.51 (d, J = 5.7 Hz, 2H, arene), 2.84 (d, J = 7.4 Hz, 2H, CH_2), 2.56 (d, J = 7.4 Hz, 2H, CH_2), 2.11 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMF-d_7) δ_{ppm} 181.3, 156.5, 153.7, 139.2, 139.1, 124.8, 101.3, 88.5, 82.0, 80.4, 38.3, 36.5, 19.2. ^{195}Pt NMR (129 MHz, DMF-d_7) δ_{ppm} 988.6. MS (ESI$^+$) *m/z*: [M–Cl]$^+$ calculated for C15H23N3O3Cl3Ru1Pt1: 695.8, found: 696.0. IR (KBr, cm^{-1}): 3443 (s), 3226 (m), 3071 (m), 2970–2872 (w), 1634 (s), 1384 (s), 1318 (m), 572 (w).

Ruthplatin **3** was synthesized using the same method as ruthplatin **1**. Brown powder. Yield: 105.0 mg, 55.6%. ^{1}H NMR (400 MHz, DMF-d_7) δ_{ppm} 8.98 (s, 1H, py), 8.89 (d, J = 5.0 Hz, 1H, py), 7.87 (d, J = 7.9 Hz, 1H, py), 7.36 (dd, J = 7.8, 5.6 Hz, 1H, py), 6.55 – 6.03 (m, 6H, $2NH_3$), 5.82 (s, 6H, arene), 2.85 (t, J = 7.3 Hz, 2H, CH_2), 2.56 (t, J = 7.3 Hz, 2H, CH_2). ^{13}C NMR (100 MHz, DMF-d_7) δ_{ppm} 180.2, 155.5, 152.7, 138.2, 138.2, 123.7, 84.5, 37.2, 35.5. MS (ESI$^+$) *m/z*: [M–Cl]$^+$ calculated for C14H21N3O3Cl3Ru1Pt1: 681.8, found: 681.8. IR (KBr, cm^{-1}): 3452 (s), 3250 (m), 3079 (w), 2960–2870 (w), 1644 (s), 1386 (s), 1321 (m), 581 (w).

3.4 Synthesis of ruthplatin 4

Synthesis of [(η^6-*p*-cym)Ru(II)(O^O)]: Silver oxalate (372.3 mg, 0.196 mmol) was added to an aqueous solution of [(η^6-*p*-cym)Ru(II)Cl(μ-Cl)$_2$]$_2$ (300.0 mg, 0.490 mmol), and was stirred for 2 h at r.t., shielded from light[3]. The obtained orange solution was frozen to dry to give an orange powder. Yield: 237.0 mg, 70.9 %.

Synthesis of ruthplatin 4: Ruthplatin **4** was synthesized by the reaction of [(η^6-*p*-cym)Ru(II)(O^O)] (40.0 mg, 0.117 mmol) with PPA-Pt(IV) (67.4 mg, 0.144 mmol) in DMF (10 mL) at r.t. in dark for 12 h. An orange solution was collected by filtration and Et_2O was added to get orange precipitate. Orange powder was obtained. Yield: 59.0 mg, 54.9 %. ^{1}H NMR (600MHz, DMF-d_7) δ_{ppm} 8.57 (s, 1H, py), 8.36 (d, J = 5.4 Hz, 1H, py), 7.95 (d, J = 7.9 Hz, 1H, py), 7.52 (dd, J = 7.7, 5.6 Hz, 1H, py), 6.63 – 6.15 (m, 6H, $2NH_3$), 5.96 (d, J = 6.0 Hz, 2H, arene), 5.74 (d, J = 6.0 Hz, 2H, arene), 2.99 – 2.85 (m, 3H, CH, CH_2), 2.61 – 2.52 (m, 2H, CH_2), 2.13 (s, 3H, CH_3), 1.33 (d, J = 6.8 Hz, 6H, $2CH_2$). ^{13}C NMR (100 MHz, DMF-d_7) δ_{ppm} 179.7, 165.0, 153.2, 150.4, 140.0, 139. 4, 125.2, 101.2, 97.5, 83.1, 80.4, 37.1, 30.9, 21.9. ^{195}Pt NMR (129 MHz, DMF-d_7) δ_{ppm} 990.50. MS (ESI$^+$) *m/z*: [M+Na]$^+$ calculated for C20H29N3O7Na1Cl2Ru1Pt1: 813.5, found: 814.8. IR (KBr, cm^{-1}): 3439 (s), 3250 (m), 3071

(w), 2966–2875 (w), 1672 (s), 1700 (s), 1385 (s), 1321 (m), 581 (w).

3.5 Synthesis of PPA-Ru(II) complexes: PPA-Ru(II) 5–7

Synthesis of PPA-Ru(II) 5: 3-Pyridinepropionic acid (24.7 mg, 0.163 mmol) was added to a solution of $[(\eta^6\text{-}p\text{-cym})Ru(II)Cl(\mu\text{-}Cl)_2]_2$ (50.0 mg, 0.082 mmol) in DCM (10 mL) and the mixture was stirred for 12 h at r.t. in dark. Then Et_2O was added to the filtered solution to get yellow precipitate, which was filtered and washed by Et_2O to give yellow powder. Yield: 65.0 mg, 86.7 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 8.95 (s, 1H, py), 8.90 (d, J = 5.7 Hz, 1H, py), 7.61 (d, J = 7.8 Hz, 1H, py), 7.23 (s, 1H, py), 5.45 (d, J = 5.9 Hz, 2H, arene), 5.22 (d, J = 5.8 Hz, 2H, arene), 3.69 – 3.35 (br, 1H, COOH), 2.98 (t, J = 7.0 Hz, 2H, CH_2), 2.71 (t, J = 7.1 Hz, 2H, CH_2), 2.08 (s, 3H, CH_3), 1.31 (d, J = 6.9 Hz, 6H, $2CH_2$). MS (ESI$^+$) *m/z*: $[M–Cl]^+$ calculated for C18H23N1O2Cl1Ru1: 421.9, found: 421.9. IR (KBr, cm^{-1}): 3456 (s), 3034 (w), 2981–2871 (w), 1639 (s), 1407 (s), 1384 (m).

PPA-Ru(II) **6** was synthesized using the same method with DMF as the solvent in the reaction. PPA-Ru(II) **6** was obtained as dark-yellow powder. Yield: 27.7 mg, 44.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 9.00 (s, 1H, py), 8.95 (d, J = 5.7 Hz, 1H, py), 7.65 (d, J = 7.7 Hz, 1H, py), 7.26 (s, 1H, py), 5.68 (t, J = 5.6 Hz, 2H, arene), 5.56 (s, 1H, arene), 5.31 (d, J = 5.7 Hz, 2H, arene), 3.05 – 2.98 (m, 2H, CH_2), 2.73 (t, J = 7.1 Hz, 2H, CH_2), 2.17 (s, 3H, CH_3). MS (ESI$^+$) *m/z*: $[M–Cl]^+$ calculated for C18H23N1O2Cl1Ru1: 379.8, found: 379.9. IR (KBr, cm^{-1}): 3457 (s), 3068 (w), 2965–2864 (w), 1639 (s), 1408 (s), 1384 (m).

PPA-Ru(II) **7** was synthesized using the same method with DMF as the solvent in the reaction. PPA-Ru(II) **7** was obtained as brown powder. Yield: 31.2 mg, 31.2%. ^{1}H NMR (300 MHz, $CDCl_3$) δ_{ppm} 8.97 (s, 1H, py), 8.95 – 8.90 (m, 1H, py), 7.66 (d, J = 7.5 Hz, 1H, py), 7.26 (s, 1H, py), 5.68 (s, 6H, arene), 2.98 (s, 2H, CH_2), 2.73 (t, J = 7.0 Hz, 2H, CH_2). MS (ESI$^+$) *m/z*: $[M–Cl]^+$ calculated for C14H15N1O2Cl1Ru1: 365.8, found: 366.0. IR (KBr, cm^{-1}): 3457 (s), 3068 (w), 2931–2864 (w), 1634 (s), 1434 (s), 1385 (m).

3.6 Synthesis of PPA-Ru(II) 8

PPA-Ru(II) **8** was synthesized by the reaction of $[(\eta^6\text{-}p\text{-cym})Ru(II)(O\text{^}O)]$ (50.0 mg, 0.147 mmol) with 3-pyridinepropionic acid (22.1 mg, 0.147 mmol) in DCM (10 mL) at r.t.. After 4 h, an orange solution was obtained and Et_2O was added to get orange precipitate. Orange powder was collected. Yield: 35.0 mg, 46.4%. ^{1}H NMR (400 MHz, $CDCl_3$) δ_{ppm} 8.50 (s, 1H, py), 8.33 (d, J = 5.5 Hz, 1H, py), 7.69 (d, J = 7.9 Hz, 1H, py), 7.30 (d, J = 5.8 Hz, 1H, py), 5.59 (d, J = 6.0 Hz, 2H, arene), 5.38 (d, J = 6.0 Hz, 2H, arene), 2.95 (t, J = 6.9 Hz, 2H, CH_2), 2.87-2.60 (m, 1H, COOH), 2.84 (m, 1H, CH), 2.68 (t, J = 7.0 Hz, 2H, CH_2), 2.10 (s, 3H, CH_3), 1.32 (d, J = 6.9 Hz, 6H, $2CH_2$). ^{13}C NMR (100 MHz, DMF-d_7) δ_{ppm} 173.3, 165.5, 153.4, 150.5, 139.0, 139.0, 125.4, 102.2, 97.2, 82.3, 80.7, 34.8, 30.9, 28.1, 22.5, 17.9. MS (ESI$^+$) *m/z*: $[M+H]^+$ calculated for C20H24N1O6Ru1: 475.5, found: 476.0. IR (KBr, cm^{-1}): 3442 (s), 3067

(w), 2968–2875 (w), 1672 (s), 1700 (s), 1403 (s), 1385 (m).

4. Cytotoxicity test

4.1 Cell lines and cell culture conditions

A2780 and A2780cisR cells were cultured in RPMI 1640 with 10% FBS and 100 units penicillin/streptomycin. HL-60 cells were cultured in phenol-red free RPMI-1640 with 10% FBS and 100 units penicillin/streptomycin. HeLa, MCF-7, A549, A549cisR and Hs27 cells were cultured in DMEM containing 10% FBS and 100 units penicillin/streptomycin. MRC-5 cells were cultured in MEM with 10% FBS, 1% *L*-glutamine, 1% non-essential amino acids, and 1% sodium pyruvate. Cisplatin-resistant cells, A549cisR and A2780cisR, were generated from their parental A549 or A2780 cells following the previous reports[4]. Briefly, A549 or A2780 cells were cultured in complete medium containing 0.5 μg/mL cisplatin at the beginning for the first screening, and the remaining cells were cultured in complete medium containing 1.0 μg/mL cisplatin for at least 4 weeks until the resistance was obtained. All cells were incubated at 37 ºC in 5% CO_2.

4.2 Cytotoxicity test

MTT assay was used to evaluate the cytotoxicity of the compounds against A2780, A2780cisR, A549, A549cisR, HeLa, and MCF-7 cells. Cells were seeded in 96-well plates at a density of 1,500 cells per well (for A2780, A549, HeLa, and MCF-7) or 2,500 cells per well (for A2780cisR, A549cisR, MRC-5 and Hs27) and incubated till their confluency reached about 30%. Medium containing different concentrations of compounds was added to each well. After 72 h incubation, the original medium was removed and 0.2 mL FBS-free medium containing 1 mg/mL MTT was added to each well. After staining for 4 h, the medium was replaced by DMSO (0.2 mL per well). The absorbance was measured at 570 nm and 730 nm. Cells incubated with medium only were set as controls. The cytotoxicity of complexes towards HL-60 cells was assessed by MTS assay. HL-60 cells were seeded in 96-well plates at a density of 40,000 cells per well using phenol-red free medium. Different concentrations of compounds were added. After 72 h incubation, MTS solution (0.2 mg/mL) together with phenazine methosulfate (PMS, 0.0046mg/mL) was added. The absorbance was determined at 490 nm and 630 nm after 4 h incubation at 37 ºC in 5% CO_2.

4.3 3D multicellular spheroid formation

Round bottom 96-well plates were used for 3D tumor spheroid culture. Poly(2-hydroxyethyl methacrylate) (Poly-HEMA) was prepared in 95% ethanol at 50 mg/mL and 50 μL was added to each well. After drying, the plates were sterilized under UV light in a safety cabinet for 2 h before use.

MCF-7 cells were seeded on the lid of a 96-well plate by adding a 10 μL droplet of cell suspension containing 2.5×10^3 cells in each well. Meanwhile, 100 μL fresh medium was added

to each well. The lid was carefully located on the plates and the plates were incubated overnight. On the second day, the droplets on the lid were carefully spun down at 1,000 rpm for 10 min. The cells were incubated for one or two more days, and round spheroids of similar size can be observed.

4.4 Cytotoxicity assay using spheroids

The cytotoxicity of ruthplatin **1** in 3-D spheroids was evaluated by calcein AM and propidium iodide (PI) double staining. Spheroids were treated with 1, 5, and 20 μM of ruthplatin **1** or cisplatin when the diameter reached around 530 μm, and exposed to those compounds for 1, 2, or 3 days. In each day the spheroids were stained with calcein AM (excitation at 488 nm, emission at 515 nm) and PI (excitation at 535 nm, emission at 617 nm) before laser confocal microscopy (Leica SPE) observation.

5. Cell cycle arrest

A2780 cells and A2780cisR were seeded in 6-well plates at a density of 3×10^5 cells per well. After attaching overnight, cells were treated with 0.5, 2, and 5 μM of cisplatin, ruthplatin **1**, cisplatin + PPA-Ru(II) **5** for 6 h. Then, fresh medium was used to replace the medium containing compounds. Cells were further incubated for 18 h and harvested by trypsinization, followed by washing twice with ice-cold PBS and fixed in 5 mL 70% ethanol overnight. Before analysis, cells were spun down, washed twice again with PBS, and stained with PI solution (20 μg/mL, containing 0.1% Triton X-100 and 200 μg/mL RNase A, pH 7.4) for 15 min at 37 ºC. Samples were finally analyzed by flow cytometer (BD FACS Calibur).

6. Replication inhibition

A2780 cells were seeded in 6-well plates and allowed to grow to 50% confluency. Cells were treated with complexes for 6 h and the medium was changed to full medium with further incubation for 18 h. Cells were subsequently labeled with bromodeoxyuridine (BrdU) (10 μM in PBS buffer) for 45 minutes, collected by trypsinization, washed, and fixed by 70% ice-cold ethanol overnight. Cells were pelleted and treated with 2N HCl/0.5% Triton X-100 for 30 minutes at room temperature to permeabilize the cell and denature the DNA. Cells were pelleted and resuspended in 0.1 M sodium tetraborate (pH 8.5) for 2 minutes. Then, cells were washed once with PBS/1% BSA and labeled with anti-BrdU-Alexa 488 (Life tech.) for 45 min at r.t. Cells were pelleted and resuspended in PBS containing 10 μg/mL RNase A and 20 μg/mL PI solution for 30 minutes. For flow cytometric measurement, Alexa 488 and PI were both excited at 488 nm and the emissions were collected at 530/30 nm and 585/42 nm for Alexa 488 and PI, respectively.

7. Apoptosis

A2780 and A2780cisR cells were seeded in 6-well plates at a density if 2×10^5 cells per well

and allowed to attach overnight. Medium containing 4 μM of cisplatin, ruthplatin **1**, and cisplatin + PPA-Ru(II) **5** was added to the cells. After 24 h treatment, cells were collected by trypsinization and washed twice in PBS. Then the cell pellets were washed once and resuspended in Annexin-V binding buffer at the density of 1×10^6 cells per mL for staining. A volume of 5 μL of FITC Annexin-V and 1 μL of PI solution were added according to the manufacturer's protocol. The samples were analyzed by a flow cytometer (BD FACS Calibur) after 15 min staining at r.t..

8. Antimetastatic properties

MDA-MB-231 cells (280,000 per well) were seeded in 700 μL media in 24-well plates and allowed to attach and grow to form a confluent monolayer. Each well of the plates was marked with a horizontal line passing through the center of bottom in advance. Wounds were created perpendicular to the lines by 20 μL tips, and unattached cells were removed by washing with PBS (pH 7.4). Calcium AM (1 mM stock solution in DMSO with 1:2000 dilution in PBS) was used to stain cells. Cells were washing with PBS for three times after staining. Complexes in DMEM with 1% FBS was added and cells incubated at 37 ºC under 5% CO_2 for imaging. DMEM with 1% FBS was used to suppress cell proliferation. Images were captured at t= 0, 12, and 24 h at the same position of each well. Experiments were repeated for at least three times. For A549, the cell density was 250,000 per well. The widths of the wounds at different time points were measured by the LAS AF Lite software.

Supplementary Figures

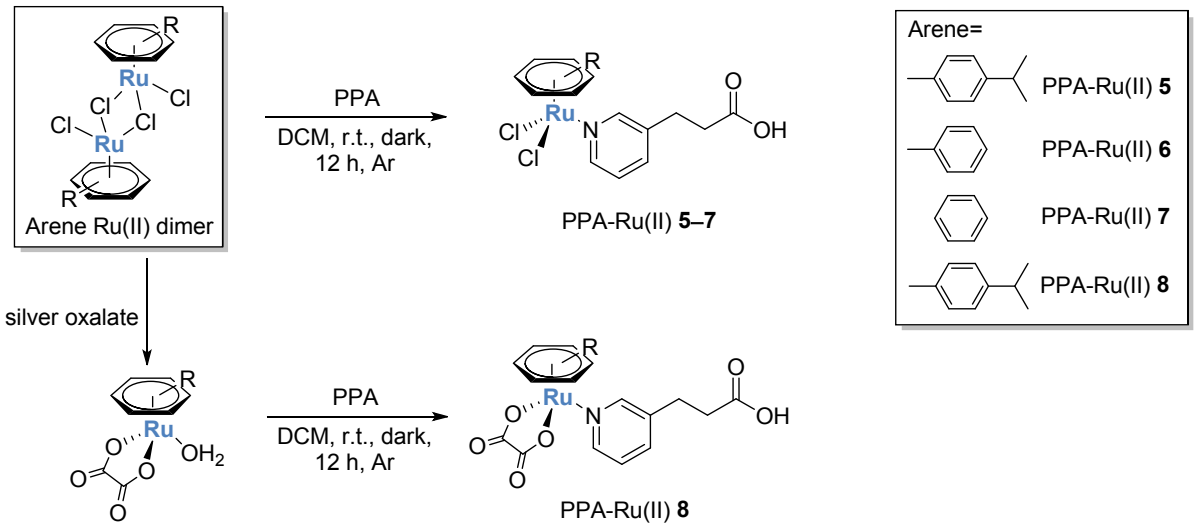

Figure S1. Synthetic routes of PPA-Ru(II) **5**–**8**.

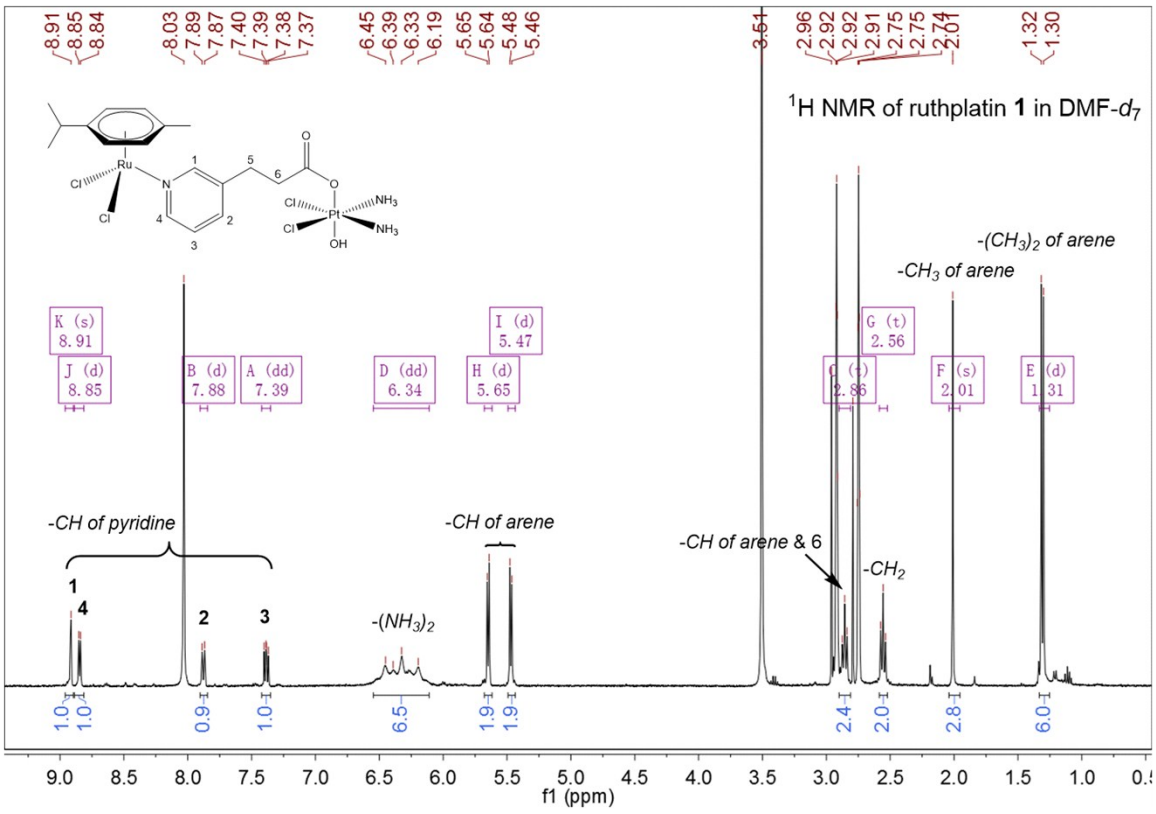

Figure S2. ^{1}H NMR (400 M) of ruthplatin **1** in DMF-d_7.

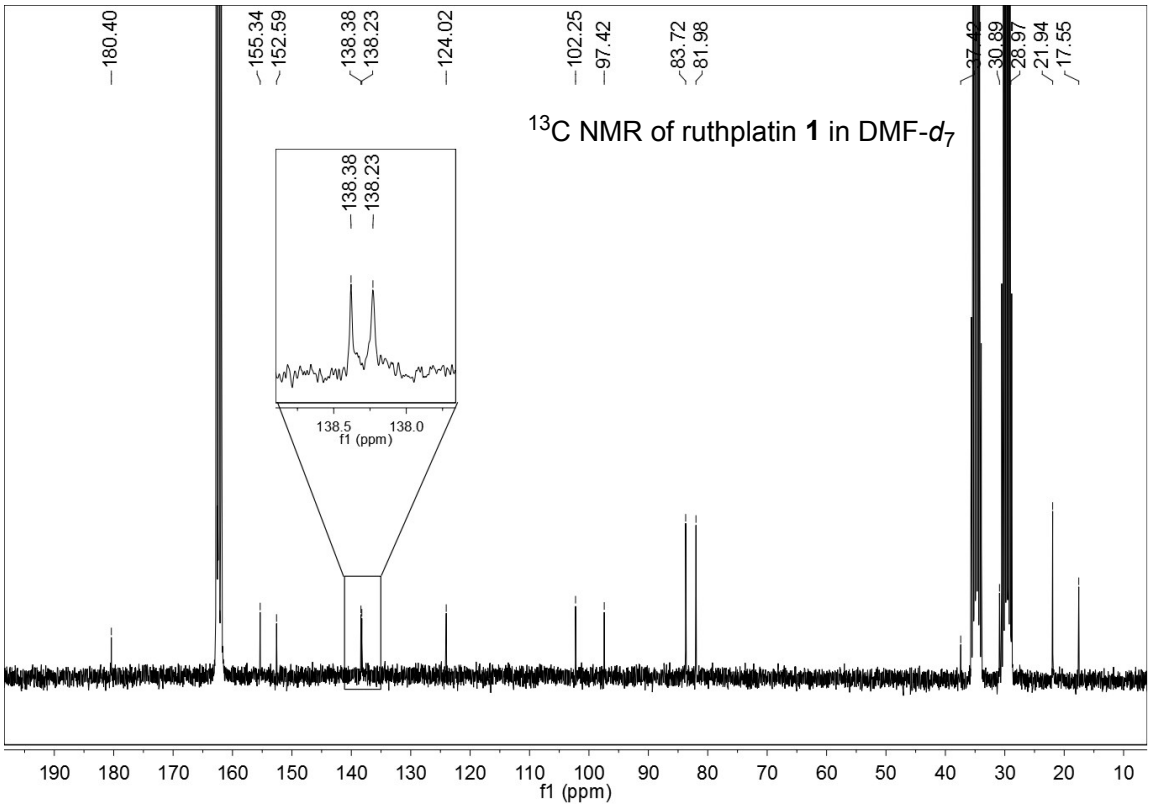

Figure S3. ^{13}C NMR (100 M) of ruthplatin **1** in DMF-d_7.

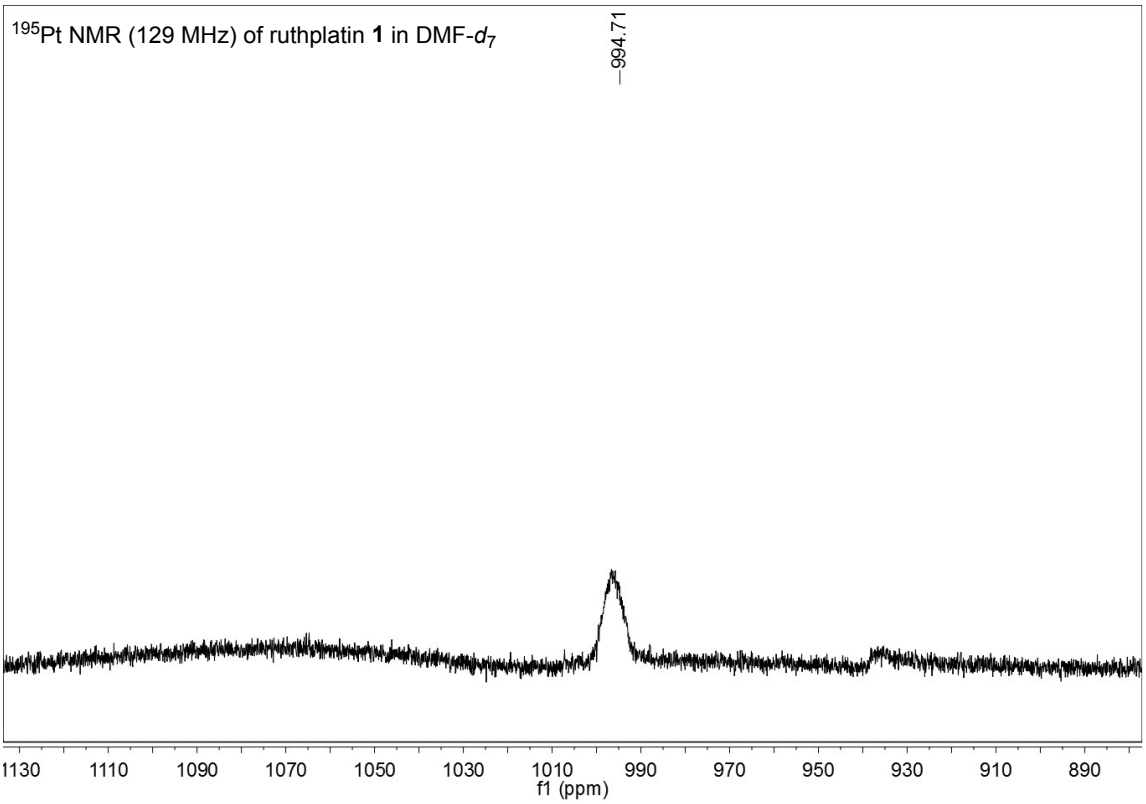

Figure S4. ^{195}Pt NMR (129 M) of ruthplatin **1** in DMF-d_7.

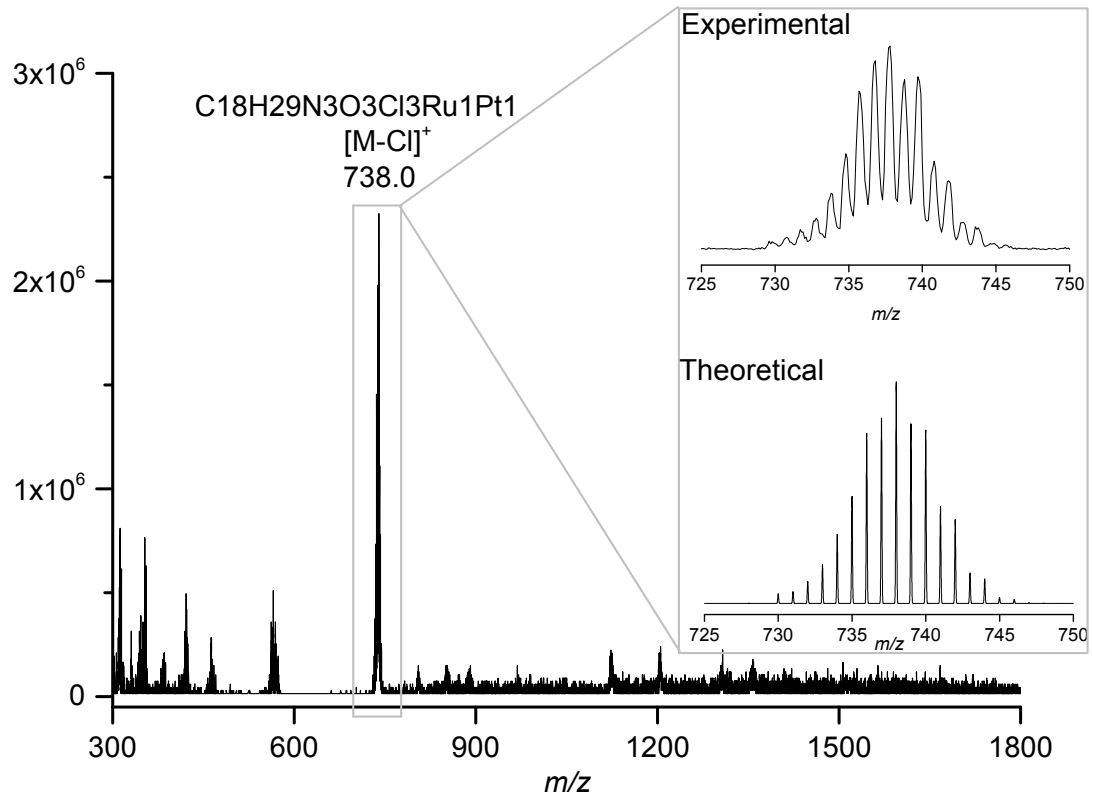

Figure S5. ESI-MS of ruthplatin **1** in methanol.

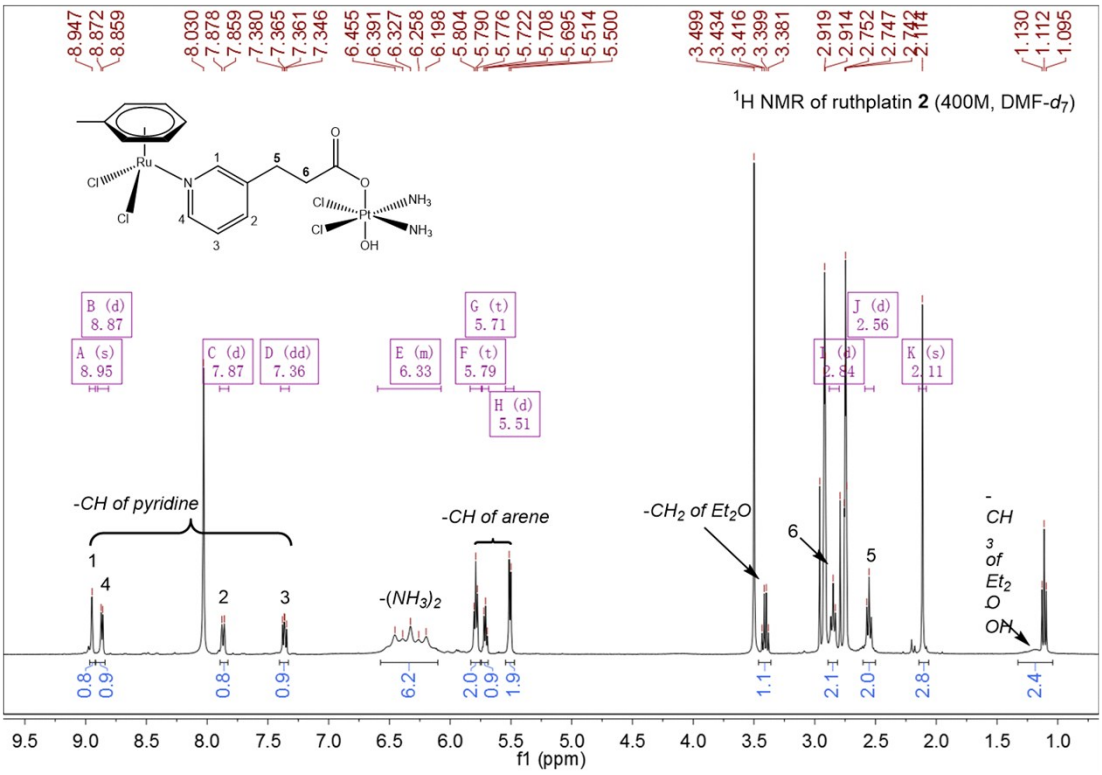

Figure S6. ^{1}H NMR (400 M) of ruthplatin **2** in DMF-d_7.

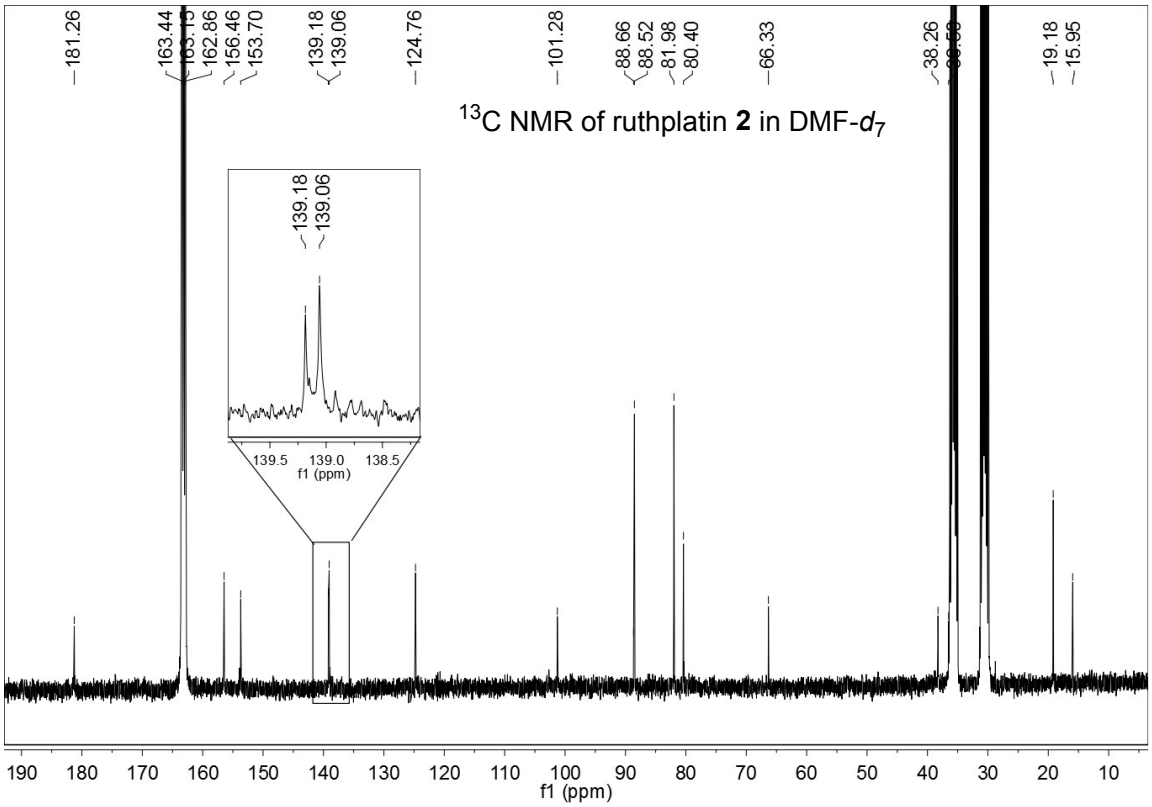

Figure S7. ^{13}C NMR (100 M) of ruthplatin **2** in DMF-d_7.

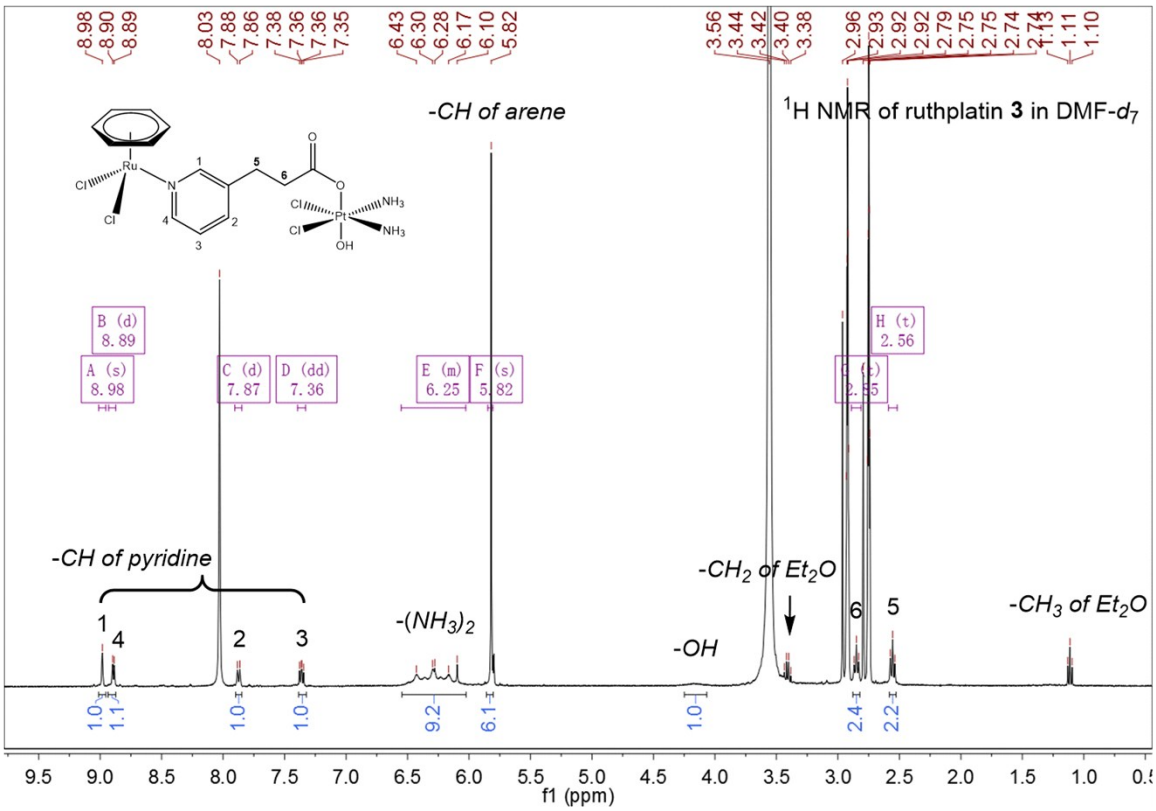

Figure S8. ^{1}H NMR (400 M) of ruthplatin **3** in DMF-d_7.

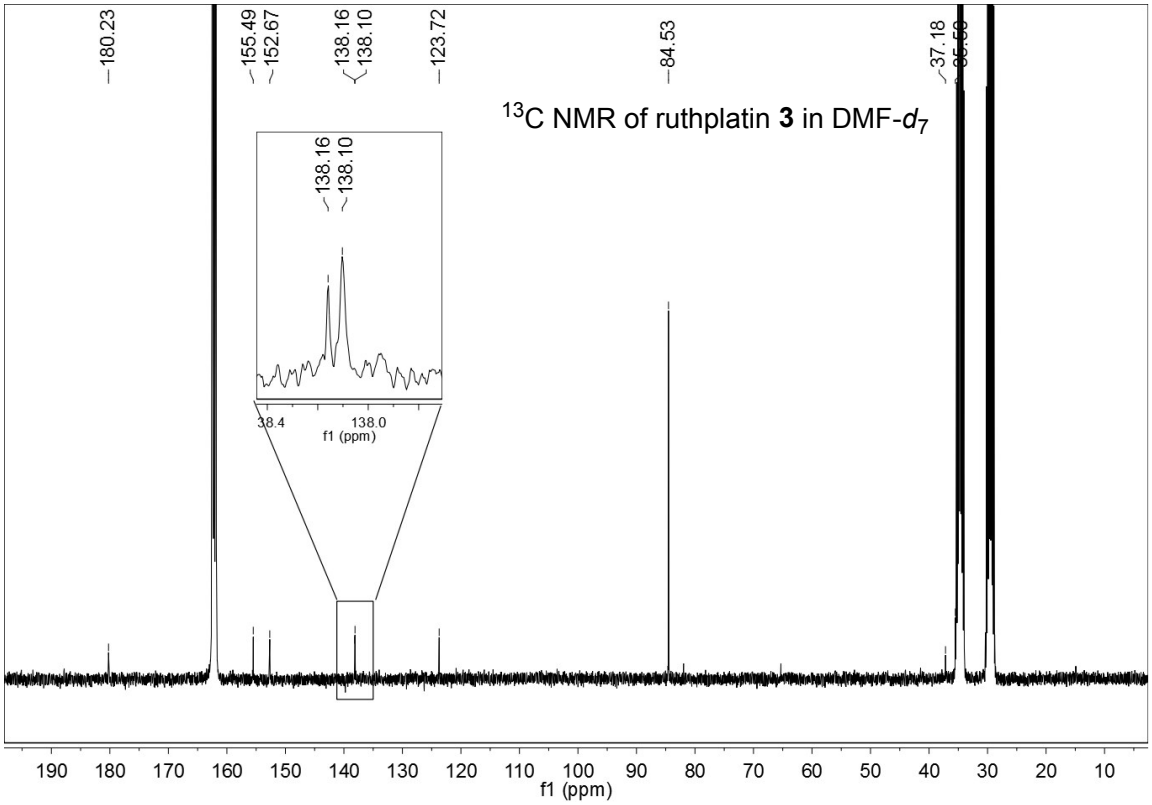

Figure S9. ^{13}C NMR (100 M) of ruthplatin **3** in DMF-d_7.

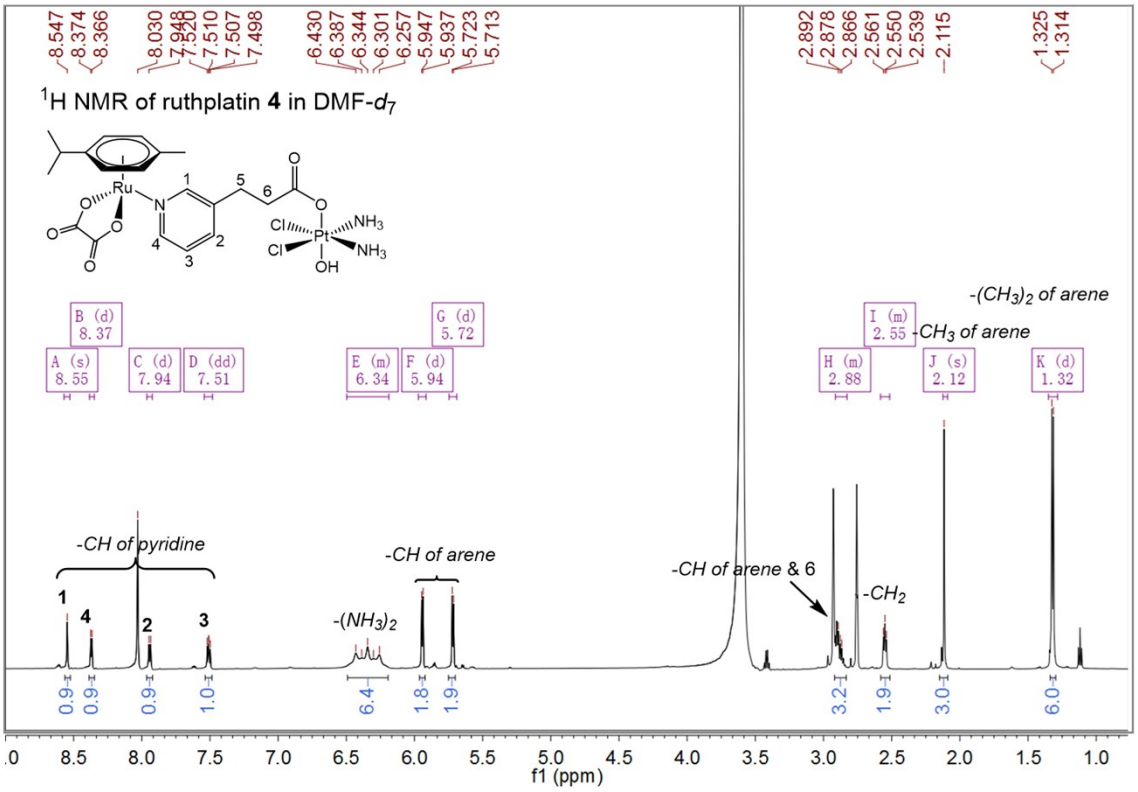

Figure S10. ^{1}H NMR (600 M) of ruthplatin **4** in DMF-d_7.

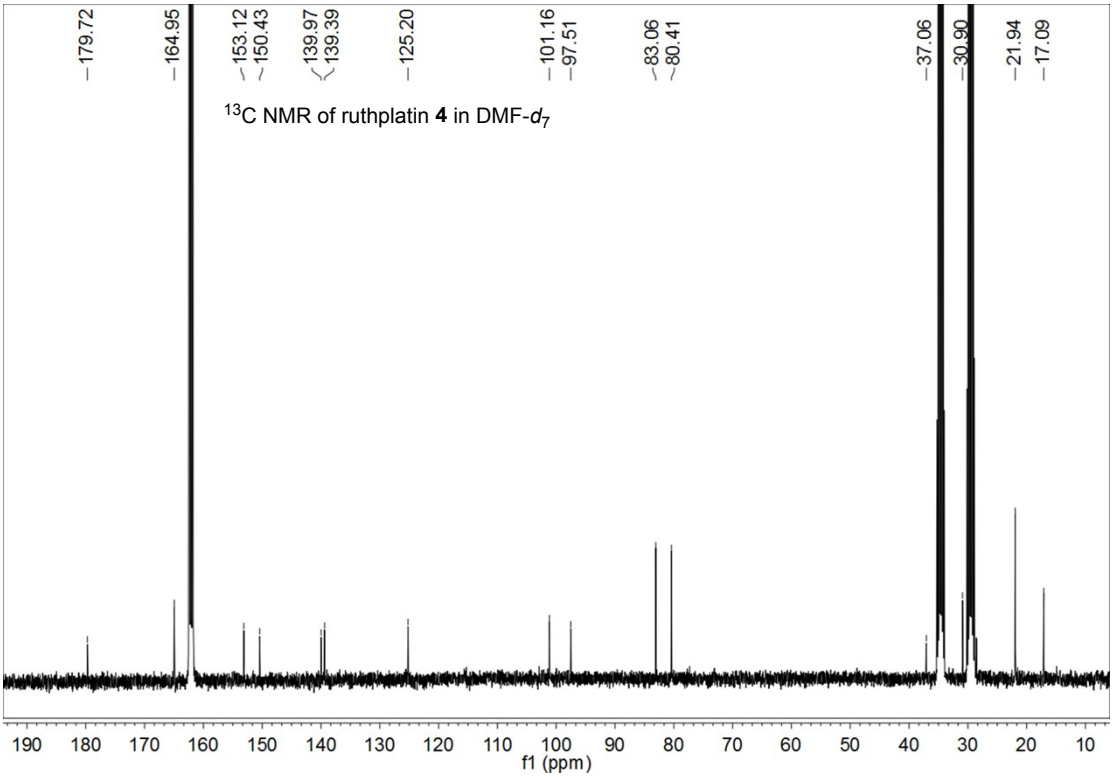

Figure S11. ^{13}C NMR (100 M) of ruthplatin **4** in DMF-d_7.

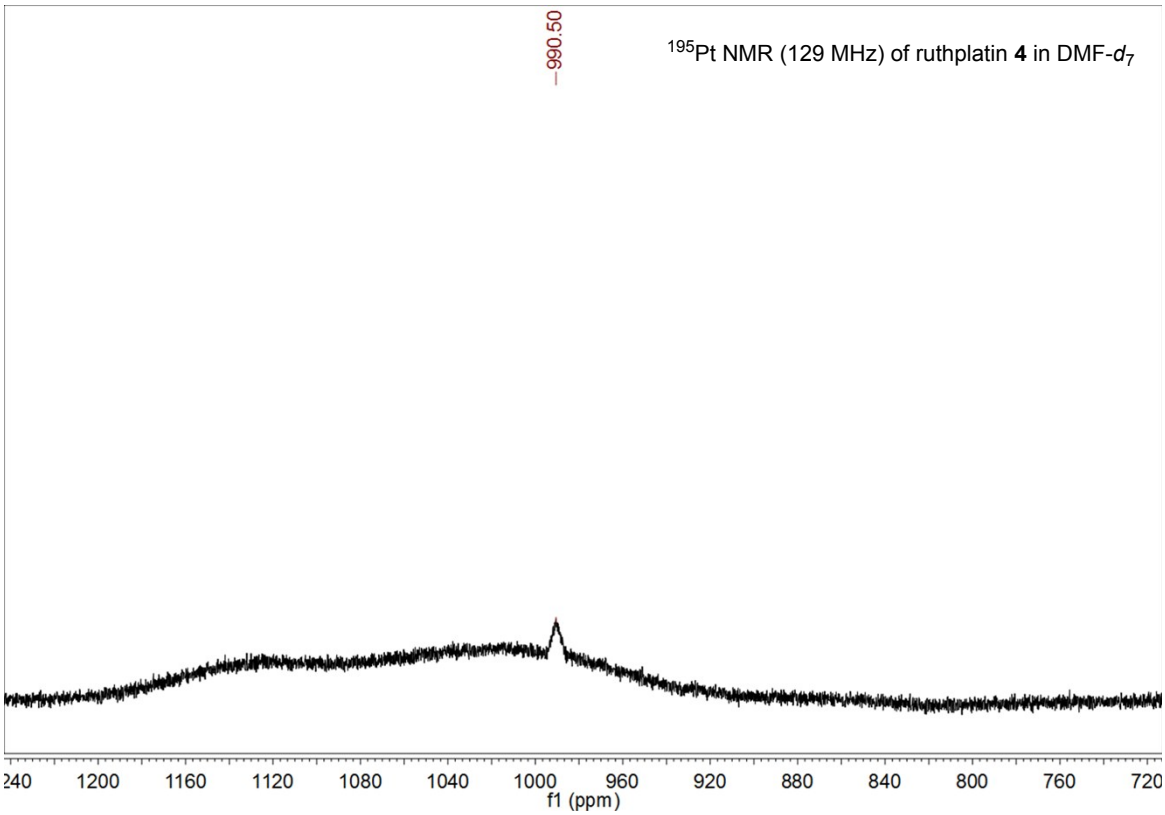

Figure S12. ^{195}Pt NMR (129 M) of ruthplatin **4** in DMF-d_7.

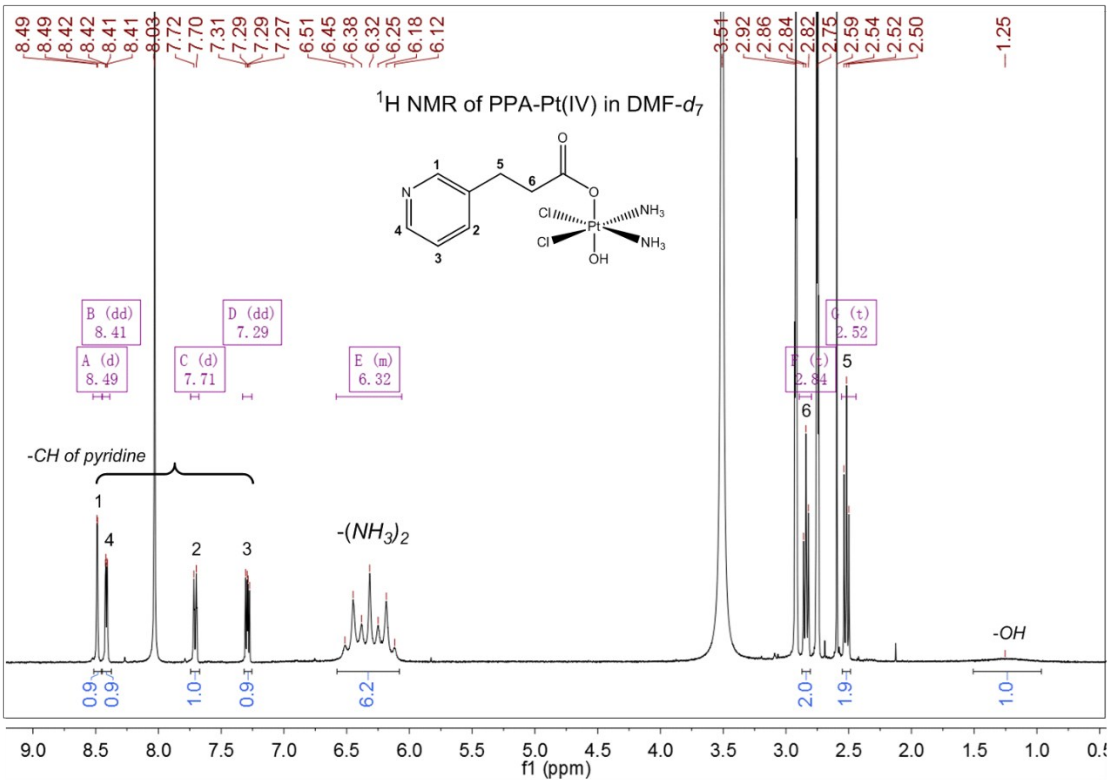

Figure S13. ^{1}H NMR (400 M) of PPA-Pt(IV) in DMF-d_7.

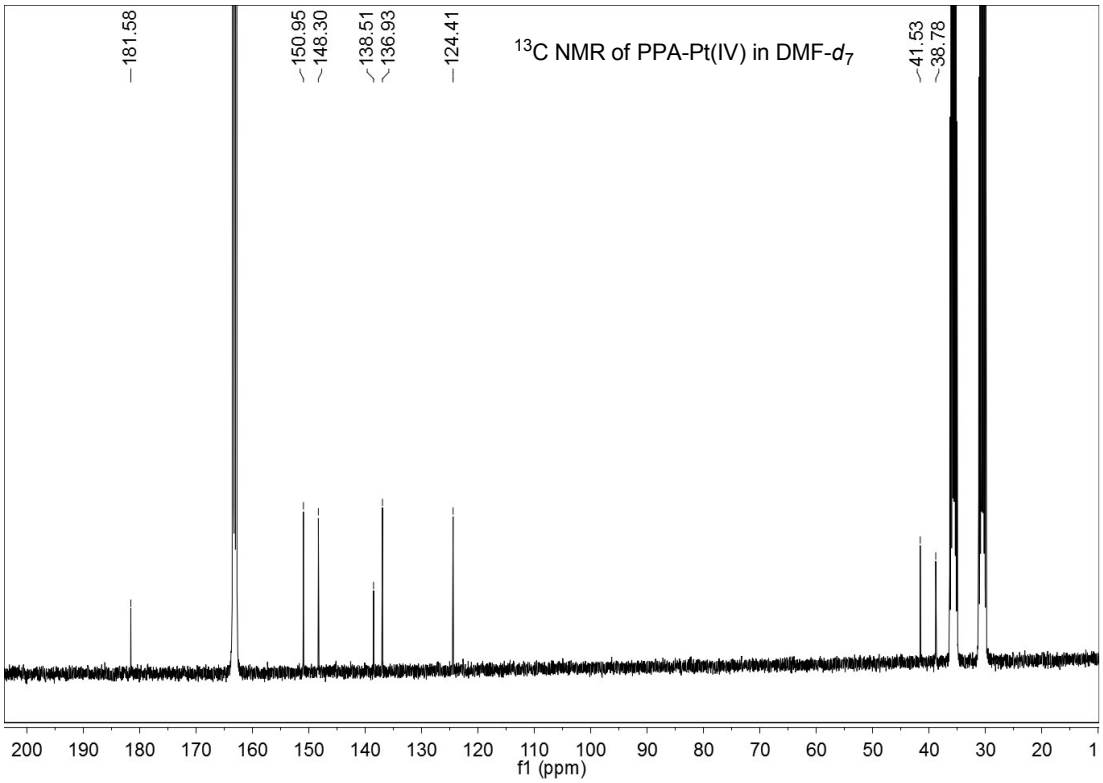

Figure S14. ^{13}C NMR (100 M) of PPA-Pt(IV) in DMF-d_7.

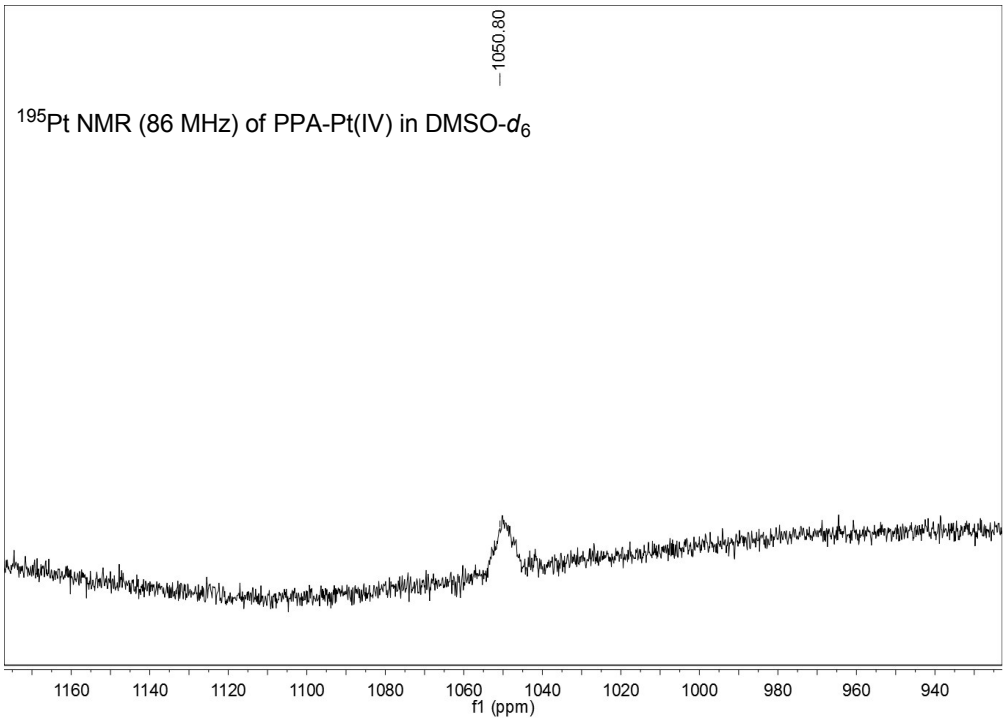

Figure S15. ^{195}Pt NMR (129 M) of PPA-Pt(IV) in DMSO-d_6.

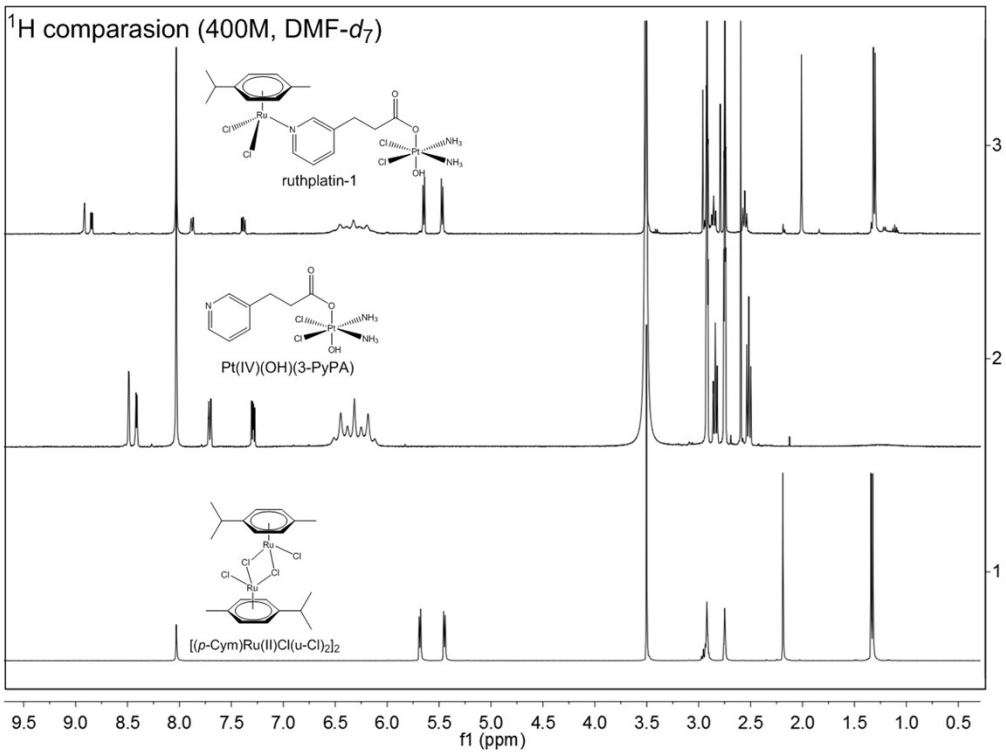

Figure S16. ^{1}H NMR spectra comparison of ruthplatin **1**, PPA-Pt(IV), and arene Ru(II) dimer in DMF-d_7.

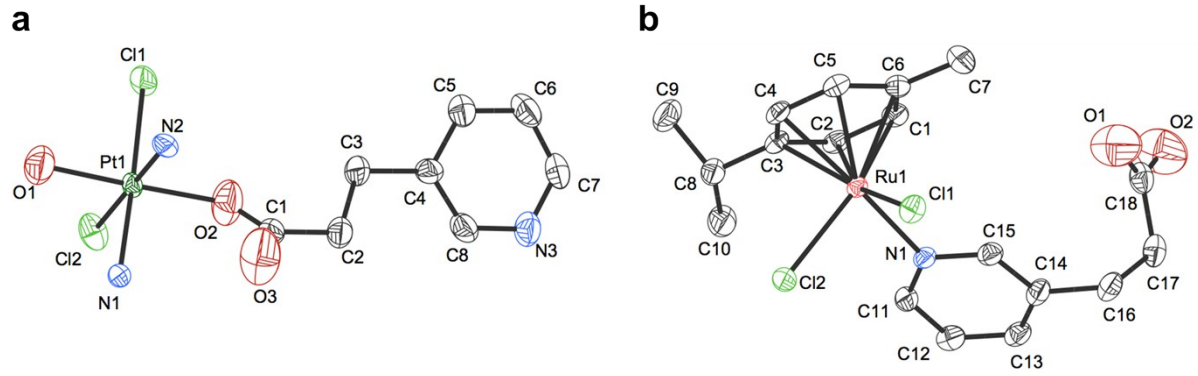

Figure S17. Crystal structures of (**a**) PPA-Pt(IV) and (**b**) PPA-Ru(II) **5**.

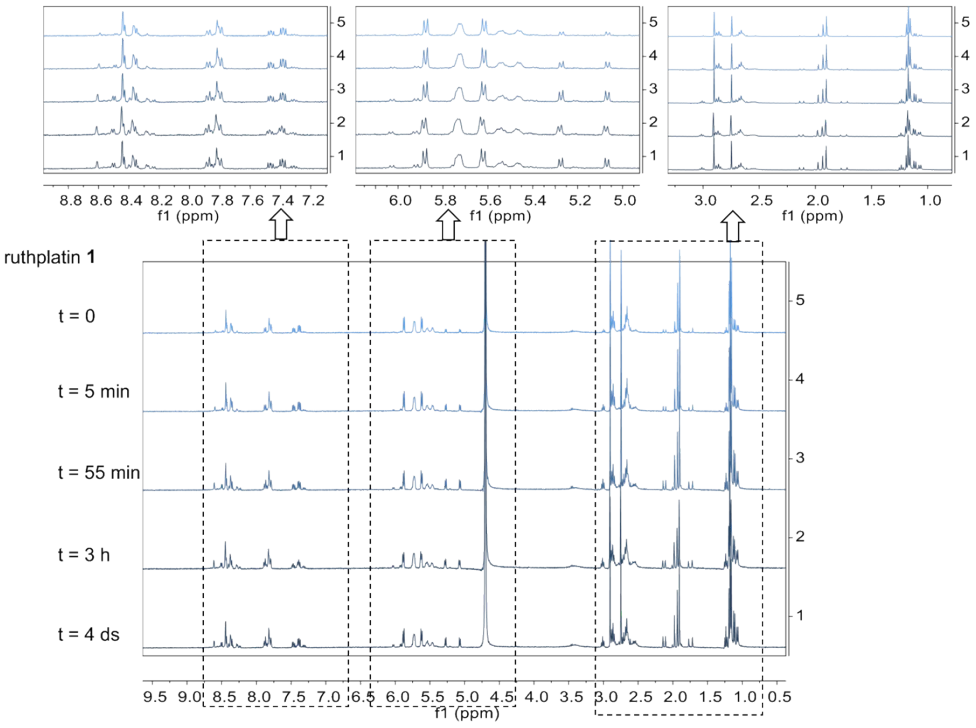

Figure S18. ^{1}H NMR spectra of ruthplatin **1** at different time points in D_2O at room temperature.

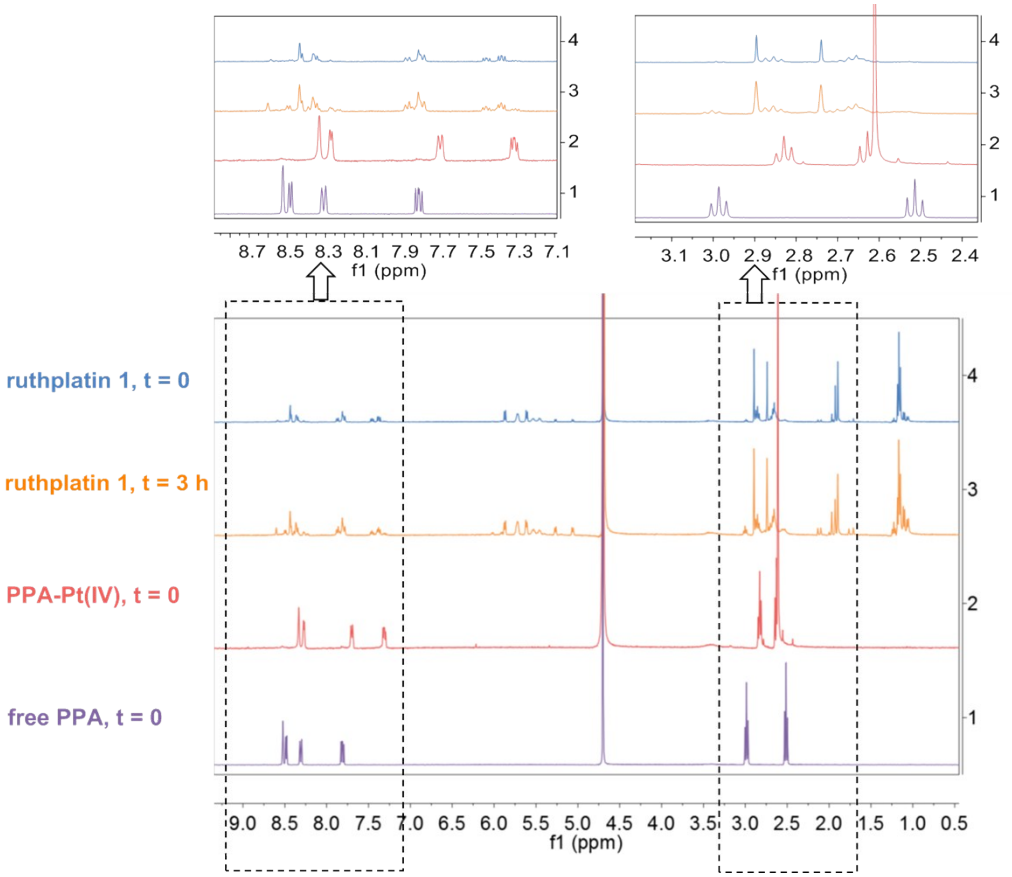

Figure S19. ^{1}H NMR spectra of ruthplatin **1**, PPA-Pt(IV), and free PPA in D_2O at room temperature.

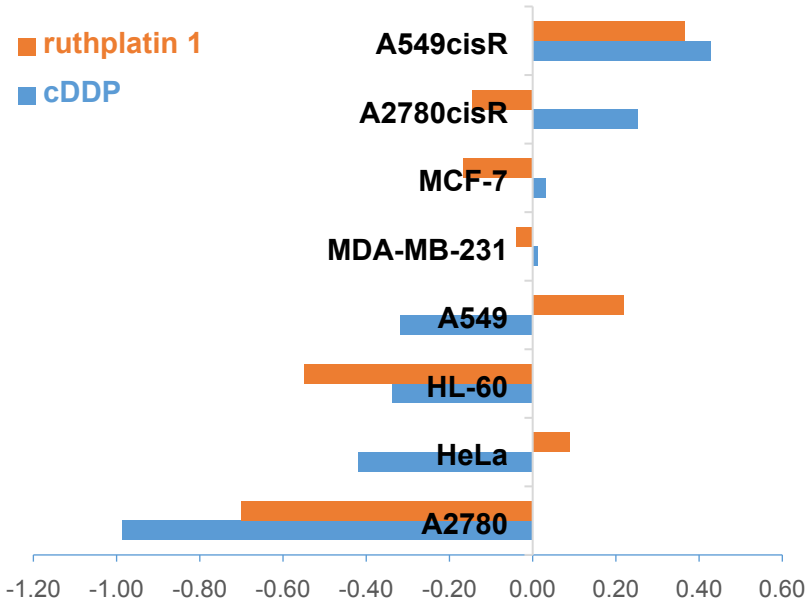

Figure S20. Comparative analysis of the cytotoxicity of cisplatin and ruthplatin **1**. The indicated values are calculated as follows: Log(IC_{50} of individual cell line) - Log(mean IC_{50}). Negative values indicate that the cell line is more sensitive than the average, whereas positive values indicate that the cell line is more resistant than the average.

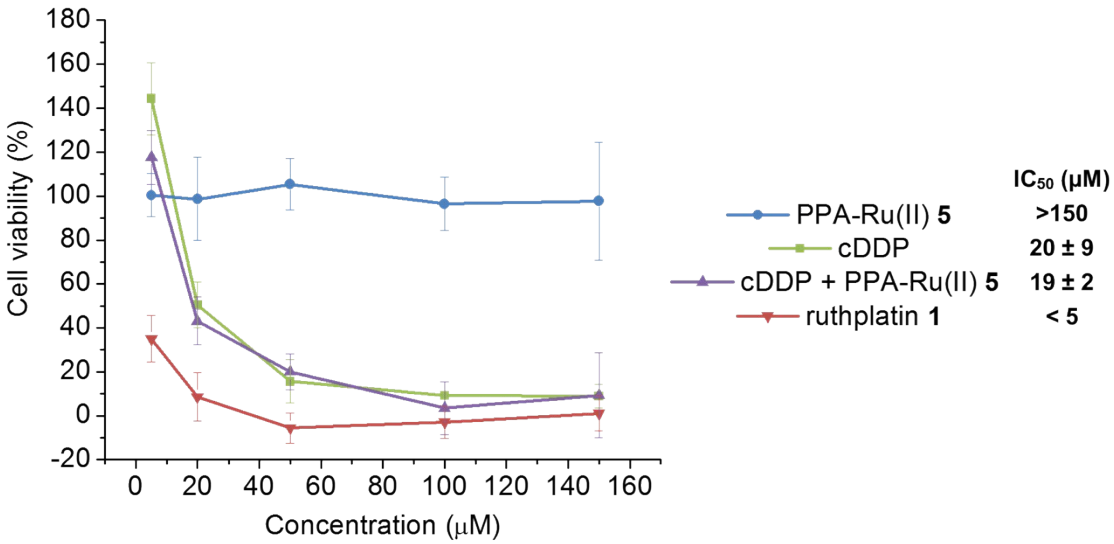

Figure S21. Cytotoxicity of ruthplatin **1** and controls in MCF-7 spheroids after 120 h treatment. Cell viability was assessed by MTS assay.

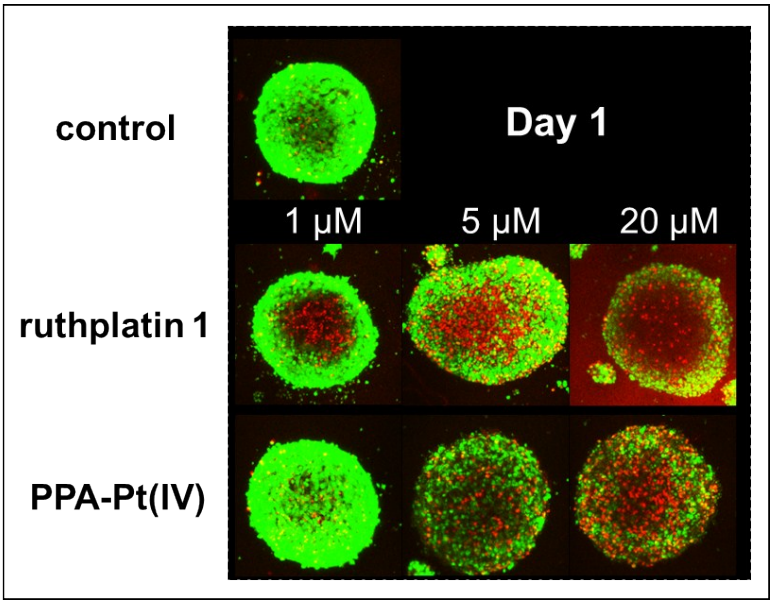

Figure S22. Images of spheroids double stained with calcein AM and PI.

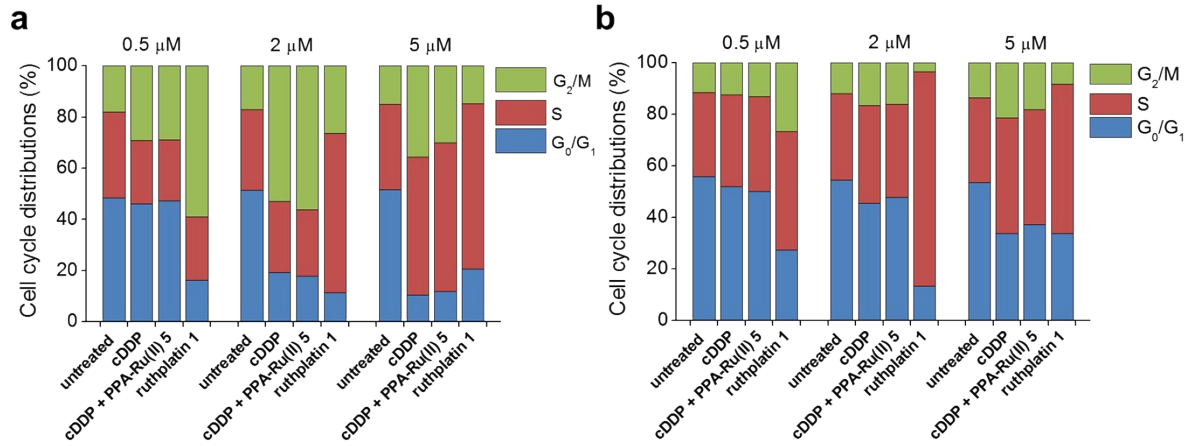

Figure S23. Cell cycle distribution upon drug treatment (0.5 μM, 2 μM, and 5 μM) for 6 h and further incubation for 18 h in (**a**) A2780 and (**b**) A2780cisR cells.

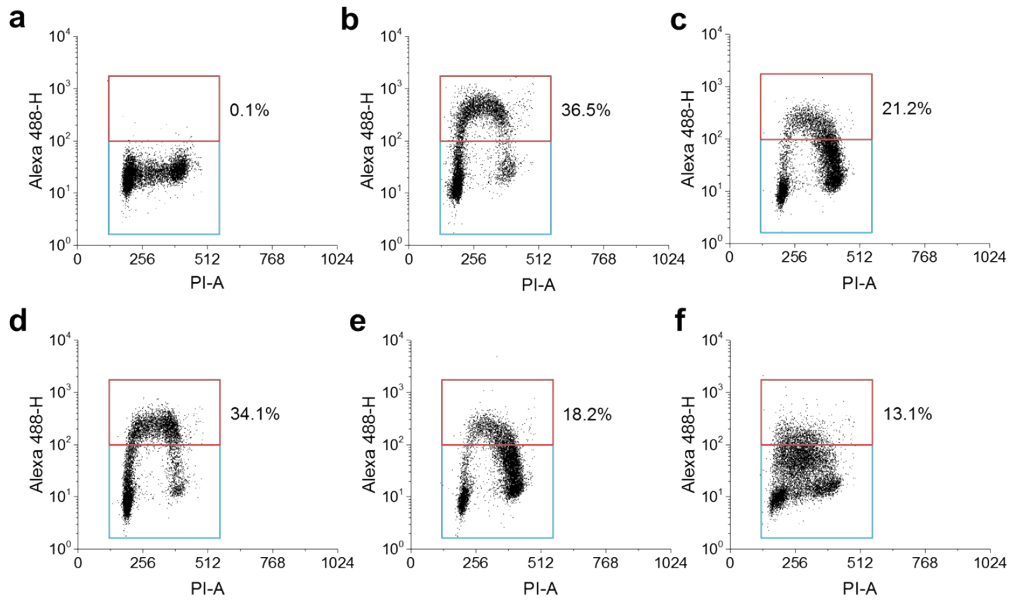

Figure S24. DNA replication analysis of A2780 cells with and without the treatment of different complexes. Cells were incubated with 10 μM BrdU before fixing and stained with anti-BrdU-Alexa 488 antibody and propidium iodide (PI). The abscissa shows cellular DNA content based on propidium iodide (PI) staining, while the ordinate gives the fluorescence resulting from incorporated and fluorescently detected BrdU. (**a**) control cells without treatment of BrdU; (**b**) control cells with treatment of BrdU; (**c**) 5 μM cDDP; (**d**) 40 μM PPA-Ru(II) **5**; (**e**) 5 μM cDDP + 5 μM PPA-Ru(II) **5**; (**f**) 5 μM ruthplatin **1**.

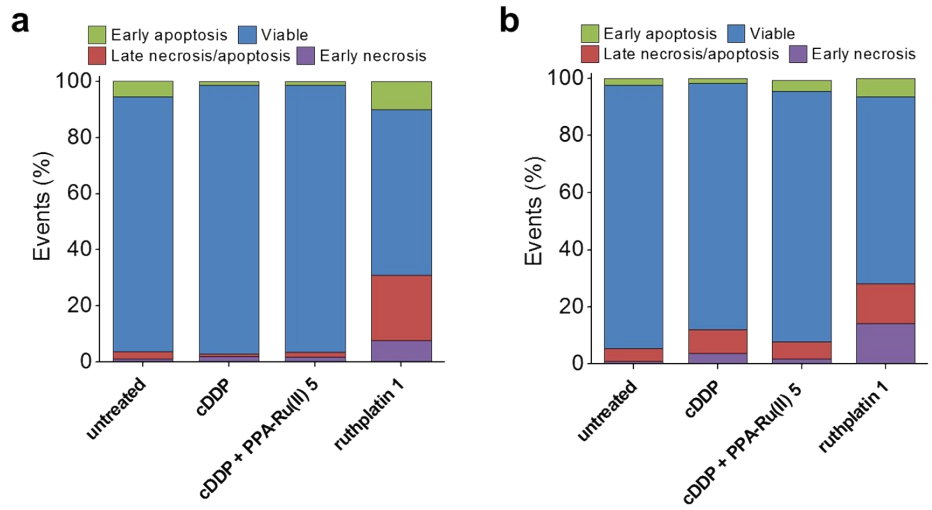

Figure S25. Flow-cytometric analysis of PI–annexin V staining of (**a**) A2780 and (**b**) A2780cisR cells treated with 4 µM complexes (by Pt) for 24 h. Viable cell cannot be stained by neither PI nor annexin V, whereas cells in early apoptotic stage can be stained with annexin V only and cells in early necrotic stage can be stained with PI only. Cells in late necrosis and late apoptosis take up both stains. Data are expressed as percentage of the cells in each condition

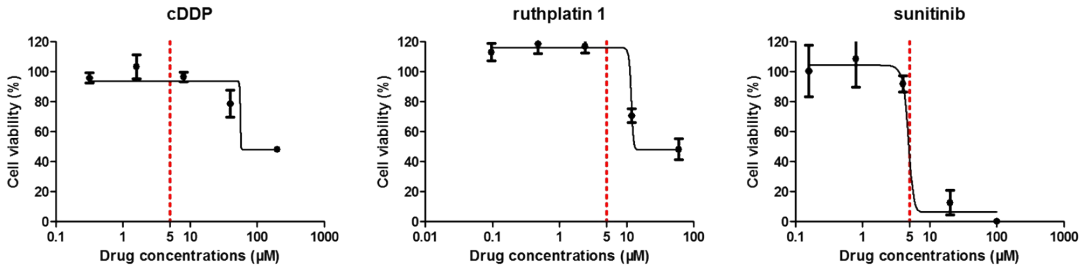

Figure S26. Cell viability of MDA-MB-231 cells after 24 h treatment with 5 µM cDDP, ruthplatin **1**, or sunitinib.

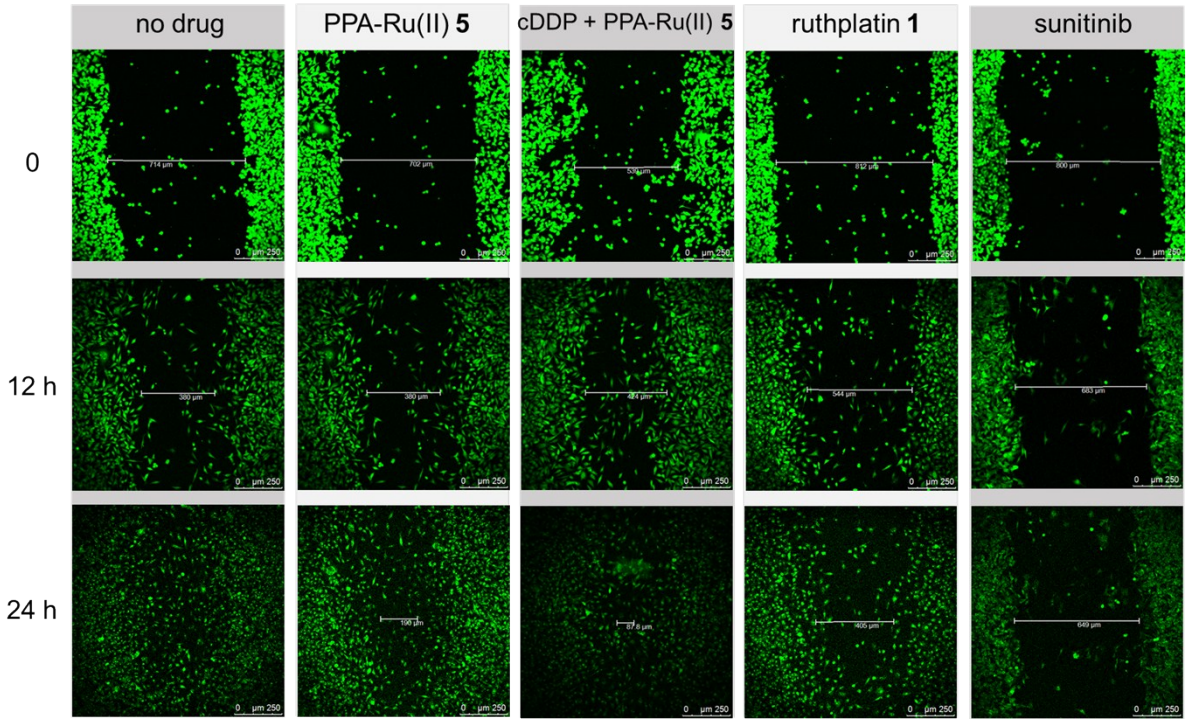

Figure S27. Migration inhibition (wound healing assay) of MDA-MB-231 cells with or without 5 μM compounds treatments for 12 h and 24 h. Typical images taken at 0, 12, and 24 h. The widths of wounds were indicated with the lines (μM).

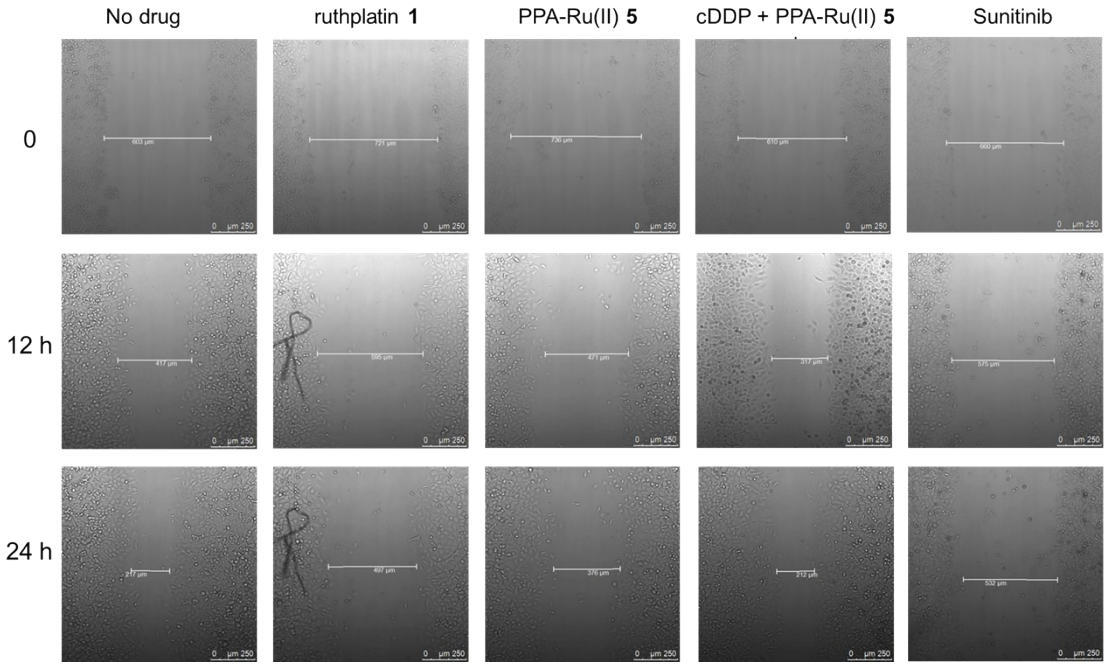

Figure S28. Typical images of migration inhibition (wound healing assay) of A549 cells with or without 5 μM complexes treatments for 12 h and 24 h. The widths of wounds were indicated with the lines. From left to right: no drug, ruthplatin **1**, PPA-Ru(II) **5**, cDDP + PPA-Ru(II) **5**, and sunitinib. Photos were taken at 0, 12, and 24 h.

Supplementary Tables

Table S1. CHN elemental analysis.

Complex	Experiential (%)			Theoretical (%) ± 0.5%			Added solvents
	C	N	H	C	N	H	
ruthplatin **1**	27.31	6.00	3.910	27.69	5.76	4.35	+ 0.5 DMF + 2.0 H_2O
ruthplatin **2**	24.66	6.04	3.508	24.64	5.75	3.17	----
ruthplatin 3	22.76	6.56	3.243	23.30	6.14	3.72	+ 0.5 DMF + 2.5 H_2O
ruthplatin **4**	30.15	5.35	4.375	30.24	5.74	4.19	+ 0.5 DMF + 1.5 H_2O
PPA-Ru(II) **5**	46.80	3.59	5.739	46.57	4.18	5.51	+0.5 DMF + 0.5 H_2O
PPA-Ru(II) **6**	40.99	3.97	4.706	41.39	4.39	4.95	+0.5 DMF + 1.5 H_2O
PPA-Ru(II) **7**	38.36	4.82	7.405	38.56	4.35	4.91	+ 0.5 DMF + 2.5 H_2O
PPA-Ru(II) **8**	48.98	3.195	5.508	48.78	2.84	5.12	+ H_2O
PPA-Pt(IV)	20.94	8.11	3.457	20.79	7.66	3.49	+0.5 DMSO + 0.5 DCM
silver oxolate	7.87	0	0.282	7.68	0	0.32	+0.5 H_2O
CymRu(II)(O^O)H_2O	41.71	0.15	4.089	42.23	0	4.73	----

Table S2. Solubility of ruthplatin **1**–**4**, PPA-Pt(IV), PPA-Ru(II) **5**, **8** and cDDP in water at room temperature.

Complexes	Solubility[a] (mM)
ruthplatin **1**	5.0
ruthplatin **2**	8.0
ruthplatin **3**	2.7
ruthplatin **4**	14.6
PPA-Ru(II) **5**	4.1
PPA-Ru(II) **8**	9.9
PPA-Pt(IV)	1.6
cDDP	4.8

[a] Solubility was measured by adding Milli-Q water to a certain amount of complex and vortex, and the supernatant was collected by centrifuge. The Pt/Ru level in the supernatant was detected by ICP-OES, and the solubility was expressed as Pt content (for ruthplatin **1**–**4**, PPA-Pt(IV) and cDDP) or Ru content (PPA-Ru(II) **5** and **8**). cDDP was tested as a reference.

Table S3. Selected bond lengths (Å) and angles (°) for PPA-Pt(IV)

Bond lengths (Å)		Angles (°)	
Pt1–O1	1.987(4)	O1–Pt1–O2	177.54(17)
Pt1–O2	2.038(4)	O1–Pt1–N1	86.42(19)
Pt1–N1	2.042(5)	O1–Pt1–N2	89.0(2)
Pt1–N2	2.037(5)	O1–Pt1–Cl1	91.41(13)
Pt1–Cl1	2.3122(16)	O1–Pt1–Cl2	90.45(16)
Pt1–Cl2	2.3300(18)	O2–Pt1–N1	95.80(19)
		O2–Pt1–Cl1	86.40(14)
		O2–Pt1–Cl2	88.55(14)
		N1–Pt1–Cl1	177.58(16)
		N1–Pt1–Cl2	88.30(16)

Table S4. Selected bond lengths (Å) and angles (°) for PPA-Ru(II) **5**.

Bond lengths (Å)		Angles (°)	
Ru1–N1	2.137(4)	N1–Ru1–Cl1	85.87(10)
Ru1–Cl1	2.4186(9)	N1–Ru1–Cl2	87.61(9)
Ru1–Cl2	2.4181(9)	Cl1–Ru1–Cl2	87.85(4)
Ru1–C1	2.161(4)		
Ru1–C2	2.185(4)		
Ru1–C3	2.205(3)		
Ru1–C4	2.193(4)		
Ru1–C5	2.185(4)		
Ru1–C6	2.192(4)		

Table S5. Crystal data and structure refinement for PPA-Pt(IV).

Identification code	PPA-Pt(IV)·0.5DMSO·0.5DCM
Empirical formula	$C_{9.5}H_{20}Cl_3N_3O_4PtS_{0.5}$
Formula weight	557.76
Temperature/K	193(2)
Crystal system	triclinic
Space group	*P*-1
a/Å	8.8701(3)
b/Å	12.4970(4)
c/Å	17.2431(6)
α/°	92.445(3)
β/°	103.722(3)
γ/°	104.921(3)
Volume/Å^3	1783.24(10)
Z	4
ρ_{calc}mg/mm^3	2.078
m/mm^{-1}	8.392
F(000)	1068.0
Crystal size/mm^3	0.64 × 0.19 × 0.03
Radiation	Mo *Kα* (*λ* = 0.71073 Å)
2*θ* range for data collection	6.7 to 50°
Index ranges	-11 ≤ h ≤ 11, -16 ≤ k ≤ 16, -23 ≤ l ≤ 15
Reflections collected	14006
Independent reflections	6274 [R_{int} = 0.0241, R_{sigma} = 0.0649]
Data/restraints/parameters	6274/60/424
Goodness-of-fit on F^2	1.083
Final *R* indexes [I>=2σ (I)]	R_1 = 0.0304, wR_2 = 0.0714
Final *R* indexes [all data]	R_1 = 0.0404, wR_2 = 0.0789
Largest diff. peak/hole / e Å^{-3}	2.38/-1.61

Table S6. Crystal data and structure refinement for PPA-Ru(II) **5**.

Identification code	PPA-Ru(II) **5**
Empirical formula	$C_{18}H_{23}Cl_2NO_2Ru$
Formula weight	457.34
Temperature/K	193(2)
Crystal system	monoclinic
Space group	$P2_1/n$
a/Å	13.7506(3)
b/Å	7.06591(11)
c/Å	19.8176(4)
α/°	90.0
β/°	109.476(2)
γ/°	90.0
Volume/Å^3	1815.31(6)
Z	4
ρ_{calc}mg/mm^3	1.673
m/mm^{-1}	9.782
F(000)	928.0
Crystal size/mm^3	0.79 × 0.14 × 0.01
Radiation	Cu *K*α (*λ* = 1.54178 Å)
2*θ* range for data collection	6.88 to 143.42°
Index ranges	-15 ≤ h ≤ 16, -8 ≤ k ≤ 4, -24 ≤ l ≤ 24
Reflections collected	6890
Independent reflections	3491 [R_{int} = 0.0454, R_{sigma} = 0.0613]
Data/restraints/parameters	3491/0/221
Goodness-of-fit on F^2	1.105
Final *R* indexes [I>=2*σ* (I)]	R_1 = 0.0539, wR_2 = 0.1584
Final *R* indexes [all data]	R_1 = 0.0565, wR_2 = 0.1640
Largest diff. peak/hole / e Å^{-3}	2.94/-1.09

Table S7. Cytotoxicity of ruthplatin **1** and controls (72 h drugs incubation). All concentrations are expressed as Pt levels.

Cell lines	IC_{50} ± SD (μM)				
A2780	0.92 ± 0.3	581 ± 31	0.74 ± 0.4 (1.2)	1.4 ± 0.5 (0.7)	0.12 ± 0.02 (7.7)
RF[b]	17	NA	NA	0.5	3.6
A549	4.3 ± 0.5	819 ± 38	5.3 ± 0.4 (0.8)	ND	1.0 ± 0.2 (4.2)
RF[b]	5.6	NA	NA	NA	1.4
MDA-MB-231	9.2 ± 2	> 200	11 ± 9 (0.8)	ND	0.55 ± 0.2 (17)

[a] FI (fold increase) is defined as IC_{50}(cDDP)/IC_{50}(ruthplatin **1**)

[b] RF (resistant factor) is defined as IC_{50} in A2780cisR/IC_{50} in A2780 or IC_{50} in A549cisR/IC_{50} in A549

[c] PPA-Pt(IV) was dissolved in 0.2% DMF for MRC-5 and in 1% DMF for other tested cell lines.

Table S8. Cytotoxicity of ruthplatin **4** and PPA-Ru(II) **8** (72 h drug incubation). All concentrations are expressed as Pt levels.

Cell lines	IC_{50} ± SD (μM) (72 h MTT)			
	cDDP	PPA-Ru(II) **8**	cDDP + PPA-Ru(II) **8** (FI[a])	ruthplatin **4** (FI[a])
A2780	0.92 ± 0.3	251 ±26	0.40 ± 0.2 (2.3)	0.09 ± 0.03(11)
A2780cisR	16 ± 5	> 50	12 ± 2 (1.3)	0.19 ± 0.03 (84)
RF[b]	17	NA	1.7	3.8
A549	4.3 ± 0.5	> 100	3.3 ± 0.6 (1.3)	0.60 ± 0.09 (7.2)
A549cisR	24 ± 3	> 500	11 ± 4 (2.2)	1.0 ± 0.3 (24)
RF[b]	5.6	NA	2.3	4.2
HeLa	3.4 ± 0.1	> 100	2.7 ± 0.4 (1.2)	0.35 ± 0.04 (9.6)

[a] FI (fold increase) is defined as IC_{50}(cDDP)/IC_{50}(ruthplatin).

[b] RF (resistant factor) is defined as IC_{50} in A2780cisR/IC_{50} in A2780 or IC_{50} in A549cisR/IC_{50} in A549.

Table S9. Migration inhibition (wound healing assay) of MDA-MB-231 cells with or without 5 μM complexes treatments for 12 h and 24 h.

complexes	12 h closure ratio (%)	24 h closure ratio (%)
no drug	43±14	100
PPA-Ru(II) **5**	39±5	91±13
PPA-Ru(II) **5** + cDDP	26±8	83±15
ruthplatin **1**	23±8	46±4
sunitinib	10±3	19±3

Table S10. Migration inhibition (wound healing assay) of A549 cells with or without 5 μM complexes treatments for 12 h and 24 h.

complexes	12 h closure ratio (%)	24 h closure ratio (%)
no drug	31	64
PPA-Ru(II) **5**	36	49
PPA-Ru(II) **5** + cDDP	48	65
ruthplatin **1**	18	31
sunitinib	13	19
ruthplatin **4**	17	36
PPA-Ru(II) **8**	32	44
PPA-Ru(II) **8** + cDDP	27	40

References

1. Hall, M. D., *et al*. The cellular distribution and oxidation state of platinum(II) and platinum(IV) antitumour complexes in cancer cells. *J. Biol. Inorg. Chem*. 2003, **8**, 726-732.

2. Bennett, M. A. *et al.* 16. (η^6-Hexamethylbenzene)ruthenium complexes. *Inorg. Synth.*, John Wiley & Sons, 2007, 74-78.

3. Ang, W. H. *et al.* Development of Organometallic Ruthenium−Arene Anticancer Drugs That Resist Hydrolysis. *Inorg. Chem.* 2006, **45**, 9006-9013.

4. Liang, X.-J. *et al*. Metallofullerene nanoparticles circumvent tumor resistance to cisplatin by reactivating endocytosis. *Pro. Natl. Acad. Sci. U.S.A*. 2010, **107**, 7449-7454.