# Triple action Pt(IV) derivatives of cisplatin: a new class of potent anticancer agents that overcome resistance.

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## Electronic Supplementary Information (ESI)

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## Experimental Section

### Materials and methods

DCA anhydride, phenylbutyric acid, ibuprofen, valproic acid and aspirin, and solvents were used as received, without further purification. Oxoplatin, *ctc*-[$Pt(NH_3)_2(OH)(OAc)Cl_2$], *ctc*-[$Pt(NH_3)_2(PhB)(OAc)Cl_2$] (CPAc) and *ctc*-[$Pt(NH_3)_2(Val)(OAc)Cl_2$] (CVAc) were synthesised according a procedure reported in the literature.

The newly synthesized Pt compounds were carachterized by $^{1}H$ (Figure S1-S11) and $^{195}Pt$ NMR, HPLC and elemental analysis.

Reaction mixtures and purified products were analyzed on a Thermo Scientific UltimaMate 3000 station, equipped with a reverse-phase C18 column (Phenomenex Kinetex 250 X 4.6 mm, 5 μm, 100 Å). UV detection was set at 220 and 260 nm. The samples were eluted with a 0 – 90 % linear gradient of acetonitrile in water over 30 min, or with a 0 – 100 % linear gradient of acetonitrile in water over 15 min followed by 15 min of 100 % acetonitrile. (Table S1)

Reaction mixtures were separated on a Thermo Scientific UltimaMate 3000 station, equipped with a reverse-phase C18 column (Phenomenex Luna 250 X 21.2 mm, 10 μm, 100 Å). UV detection was set at 220 nm. The elution conditions for each product are reported in the text.

All NMR data were collected on a Bruker AVANCE IIITM HD 500 MHz spectrometer using a 500 Mhz SmartProbe. $^{195}Pt$ NMR data were collected at 107.47 MHz using sw= 217391.3 Hz aq=0.002sec and rd=0.001sec. The data were processed using MestreNova with a 300 Hz line broadening and backward linear prediction. $^{1}H$ spectra were recorded with standard parameters.

### Synthesis

**Anhydrides: general procedure.** 50 mM solution of starting free acid in chloroform were stirred overnight in the presence of 0.6 Eq of EDC. The mixture was washed three times with citric acid aqueous solution (1g/100 mL), and three times with sodiumbicarbonate aqueous solution (1g/100 mL). It was then dried on sodium sulphate, and finally evaporated to dryness under reduced pressure.

***ctc*-[$Pt(NH_3)_2(Asp)(DCA)Cl_2$] (CAD).** Oxoplatin (51.3 mg, 0.15 mmol) and aspirin anhydride (63.1 mg, 0.18 mmol, 1.2 Eq) were stirred in 4.0 mL of DMSO for 1 day at RT. The mixture

was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and dichloroacetic anhydride (143.9 mg, 0.60 mmol, 4 Eq) was added to the mixture. After 5 h at RT, the mixture turned to a bright yellow solution. It was filtered, concentrated and precipitated with a 1 : 1 mixture of diethylether and petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 19.5 min, 0-80 % linear gradient of acetontirile in water over 24 min) and then lyophiolized. Finally, it was dissolved in acetone and precipitated with ether, collected and dried. 23.7 mg were recovered, corresponding to a yield of 26 %. $^{1}$H NMR (acetone-$d_6$): 7.89 (d, 1H, Asp), 7.54 (t, 1H, Asp), 7.28 (t, 1H, Asp), 7.08 (d, 1H, Asp), 6.57 (m, 6H, $NH_3$), 6.33 (s, 1H, DCA), 2.32 ppm (s, 3H, Asp). $^{195}$Pt NMR (acetone-$d_6$): 1139 ppm. Elemental analysis calculated for $C_{11}H_{14}Cl_4N_2O_6Pt$ (*1$H_2O$): C, 21.13; H, 2.58; N, 4.48; %. Found: C, 21.34; H, 2.35; N, 4.25 %.

***ctc*-[Pt(NH$_3$)$_2$(PhB)(Asp)Cl$_2$] (CPA).** Oxoplatin (71.5 mg, 0.21 mmol) and aspirin anhydride (88.0 mg, 0.26 mmol, 1.2 Eq) were stirred in 5.0 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and phenylbutyric anhydride (195.5 mg, 0.63 mmol, 3 Eq) was added to the mixture. After one day at RT, the mixture turned to a pale yellow solution. It was filtered, concentrated and precipitate with a 1 : 1 mixture of diethyl ether and petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 15.5 min, 30-80 % linear gradient of acetonitrile in water over 20 min) and then lyophilized. Finally, it was dissolved in acetone and precipitated with ether, collected and dried. 45.0 mg were recovered, corresponding to a yield of 33 %. $^{1}$H NMR (acetone-$d_6$): 7.93 (d, 1H, Asp), 7.54 (t, 1H, Asp), 7.30 – 7.23 (m, 5H, PhB), 7.18 (t, 1H, Asp), 7.08 (d, 1H, Asp), 6.54 (m, 6H, $NH_3$), 2.67 (t, 2H, PhB), 2.33 (s, 3H, Asp), 2.32 (t, 2H, PhB), 1.86 ppm (q, 2H, PhB). $^{195}$Pt NMR (acetone-$d_6$): 1141 ppm. Elemental analysis calculated for $C_{19}H_{24}Cl_2N_2O_6Pt$ (*0.5$H_2O$): C, 35.03; H, 3.87; N, 4.30; %. Found: C, 34.75; H, 3.51; N, 4.15 %.

***ctc*-[Pt(NH$_3$)$_2$(Ibu)(DCA)Cl$_2$] (CID).** Oxoplatin (73.6 mg, 0.22 mmol) and ibuprofen anhydride (95.6 mg, 0.24 mmol, 1.1 Eq) were stirred in 7.0 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was

repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and dichloroacetic anhydride (211.1 mg, 0.88 mmol, 4 Eq) was added to the mixture. After a day at RT, the mixture turned to a bright yellow solution. It was filtered, concentrated and precipitate with a 1 : 1 mixture of diethyl ether and petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 23.6 min, 10-88 % linear gradient of acetonitrile in water over 26 min) and then lyophilized. Finally, it was dissolved in acetone, precipitated with ether, collected and dried. 10.1 mg were recovered, corresponding to a yield of 7 %. $^{1}$H NMR (acetone-$d_6$): 7.29 (d, 2H, Ibu), 7.08 (d, 2H, Ibu), 6.52 (m, 6H, $NH_3$), 6.27 (s, 1H, DCA), 3.73 (q, 1H, Ibu), 2.46 (d, 2H, Ibu), 1.86 (m, 1H, Ibu), 1.37 (d, 3H, Ibu), 0.90 (d, 6H, Ibu) ppm. $^{195}$Pt NMR (acetone-$d_6$): 1136 ppm. Elemental analysis calculated for $C_{15}H_{24}Cl_4N_2O_4Pt$ (*2.7 $H_2O$ * 1.3 DCA * 0.7 ACN): C, 25.92; H, 3.89; N, 4.24; %. Found: C, 25.55; H, 3.64; N, 4.60 %.

***ctc*-[Pt(NH$_3$)$_2$(PhB)(DCA)Cl$_2$] (CPD).** Oxoplatin (37.5 mg, 0.11 mmol) and phenylbutyric anhydride (38.3 mg, 0.12 mmol, 1.1 Eq) were stirred in 3.5 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and dichloroacetic anhydride (105.5 mg, 0.44 mmol, 4 Eq) was added to the mixture. After 2 h at RT, the mixture turned to a bright yellow solution. It was filtered, concentrated and precipitate with a 1:1 mixture of diethyl ether and petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 17.1 min, 18-90 % linear gradient of acetontirile in water over 23 min) and then lyophilized. Finally, it was dissolved in acetone, precipitated with ether, collected and dried 23.1 mg were recovered, corresponding to a yield of 36 %. $^{1}$H NMR (acetone-$d_6$): 7.28 (t, 2H, PhB), 7.24 (d, 2H, PhB), 7.18 (t, 1H, PhB), 6.50 (m, 6H, $NH_3$), 6.27 (s, 1H, DCA), 2.65 (t, 2H, PhB), 2.32 (t, 2H, PhB), 1.84 (q, 2H PhB) ppm. $^{195}$Pt NMR (acetone-$d_6$): 1141 ppm. Elemental analysis calculated for $C_{12}H_{18}Cl_4N_2O_4Pt$ (*1/6acetone): C, 24.99; H, 3.19; N, 4.66; %. Found: C, 24.85; H, 2.84; N, 4.34 %.

***ctc*-[Pt(NH$_3$)$_2$(PhB)(Ibu)Cl$_2$] (CPI).** Oxoplatin (86.5 mg, 0.26 mmol) and phenylbutyric anhydride (88.4 mg, 0.28 mmol, 1.1 Eq) were stirred in 8 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile

and ibuprofen anhydride (205.1 mg, 0.52 mmol, 2 Eq) was added to the mixture. After 2 h at RT, the mixture turned to a yellow solution. It was filtered, concentrated and precipitate with petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 14.9 min, 0-100 % linear gradient of acetontirile in water over 13 min followed by a pleatau of 100 % acetontirle for 5 min) and then lyophilized. Finally, it was dissolved in acetone, precipitated with ether, collected and dried. 67.0 mg were recovered, corresponding to a yield of 38 %. $^{1}$H NMR (acetone-$d_6$): 7.29 (d, 2H, Ibu), 7.26 – 7.22 (m, 4H, PhB), 7.17 (t, 1H, PhB), 7.07 (d, 2H, Ibu), 6.45 (m, 6H, $NH_3$), 3.70 (q, 1H, Ibu), 2.65 (t, 2H, PhB), 2.45 (d, 2H, Ibu), 2.28 (t, 2H, PhB), 1.87 (m, 1H, Ibu), 1.83 (q, 2H, PhB), 1.37 ppm (d, 3H, Ibu), 0.89 (d, 6H, Ibu) ppm. $^{195}$Pt NMR (acetone-$d_6$): 1137 ppm. Elemental analysis calculated for $C_{23}H_{34}Cl_2N_2O_4Pt$: C, 41.32; H, 5.13; N, 4.19; %. Found: C, 41.03; H, 5.14; N, 4.10 %.

***ctc*-[Pt(NH$_3$)$_2$(Val)(DCA)Cl$_2$] (CVD).** Oxoplatin (36.2 mg, 0.11 mmol) and valproic anhydride (35.1 mg, 0.13 mmol, 1.2 Eq) were stirred in 3.5 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and dichloroacetic anhydride (105.5 mg, 0.44 mmol, 4 Eq) was added to the mixture. After 2 h at RT, the mixture turned to a bright yellow solution. It was filtered, concentrated and precipitated with petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 19.8 min, 15-90 % linear gradient of acetontirile in water over 25) and then lyophilized. Finally, it was dissolved in acetone, precipitated with ether, collected and dried. 27.0 mg were recovered, corresponding to a yield of 43 %. $^{1}$H NMR (acetone-$d_6$): 6.52 (m, 6H, $NH_3$), 6.27 (s, 1H, DCA), 2.38 (m, 1H, Val), 1.55 (m, 2H, Val), 1.33 ppm (m, 2H, Val), 1.30 ppm (m, 4H, Val), 0.87 ppm (m, 6H, Val). $^{195}$Pt NMR (acetone-$d_6$): 1141 ppm. Elemental analysis calculated for $C_{10}H_{22}Cl_4N_2O_4Pt$ (*0.5ACN*0.5$Et_2O$*3$H_2O$): C, 22.87; H, 5.09; N, 5.13; %. Found: C, 22.74; H, 5.04; N, 5.47 %.

***ctc*-[Pt(NH$_3$)$_2$(Val)(Asp)Cl$_2$] (CVA).** Oxoplatin (117.0 mg, 0.35 mmol) and valproic anhydride (142.1 mg, 0.52 mmol, 1.5 Eq) were stirred in 12 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrigufation. This procedure was

repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and aspirin anhydride (239.6 mg, 0.70 mmol, 2 Eq) was added to the mixture. After 1 day at RT, the mixture turned to a yellow solution. It was filtered, concentrated and precipitate with petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 22.0 min, 15-87 % linear gradient of acetonitrile in water over 27 min) and then lyophilized. Finally, it was dissolved in acetone, precipitated with ether, collected and dried. 72.4 mg were recovered, corresponding to a yield of 33 %. $^{1}$H NMR (acetone-$d_6$): 7.94 (d, 1H, Asp), 7.54 (t, 1H, Asp), 7.29 (t, 1H, Asp), 7.08 (d, 1H, Asp), 6.51 (m, 6H, $NH_3$), 2.40 (m, 1H, Val), 2.34 (s, 3H, Asp), 1.55 (m, 2H, Val), 1.38 (m, 1H, Val), 1.35 (m, 6H, Val) , 0.88 ppm (m, 6H, Val). $^{195}$Pt NMR (acetone-$d_6$): 1141 ppm. Elemental analysis calculated for $C_{17}H_{28}Cl_2N_2O_6Pt$ (*0.4$H_2O$): C, 32.43; H, 4.62; N, 4.45; %. Found: C, 32.07; H, 4.26; N, 4.46 %.

***ctc*-[Pt(NH$_3$)$_2$(Val)(Ibu)Cl$_2$] (CVI).** Oxoplatin (65.7 mg, 0.20 mmol) and valproic anhydride (79.8 mg, 0.30 mmol, 1.5 Eq) were stirred in 7.0 mL of DMSO for 1 day at RT. The mixture was filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile and ibuprofen anhydride (236.7 mg, 0.60 mmol, 3 Eq) was added to the mixture. After 5 h at RT, the mixture turned to a pale yellow solution. It was filtered, concentrated and precipitate with petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 17.4 min, 0-100 % linear gradient of acetontirile in water over 15 min followed by a pleatau of 100 % acetontirle for 5 min). The product was lyophilized. Finally, it was dissolved in acetone, precipitated with ether, collected and dried. 38.3 mg were recovered, corresponding to a yield of 30 %. $^{1}$H NMR (acetone-$d_6$): 7.29 (d, 2H, Ibu), 7.08 (d, 2H, Ibu), 6.42 (m, 6H, $NH_3$), 3.70 (q, 1H, Ibu), 2.45 (d, 2H, Ibu), 2.34 (m, 1H, Val), 1.85 (m, 1H, Ibu), 1.53 (m, 2H, Val), 1.36 (d, 3H, Ibu), 1.34 (m, 2H, Val), 1.31 (m, 4H, Val), 0.90 (m, 6H, Ibu), 0.86 ppm (t, 6H, Val). $^{195}$Pt NMR (acetone-$d_6$): 1136 ppm. Elemental analysis calculated for $C_{21}H_{38}Cl_2N_2O_4Pt$ (*1$H_2O$): C, 37.84; H, 6.05; N, 4.20; %. Found: C, 37.68; H, 5.86; N, 3.93 %.

***ctc*-[Pt(NH$_3$)$_2$(DCA)(OAc)Cl$_2$] (CDAc).** *Ctc*-[Pt($NH_3$)$_2$(OH)(OAc)$Cl_2$] (58.7 mg, 0.16 mmol) and DCA anhydride (149.8 mg, 0.62 mmol, 4Eq) were stirred in 2.5 mL of DMF for 4 hours at RT. The mixture turned to a yellow solution. It was filtered, recovering the mother liquor that was dried under vacuum. The sticky yellow solid was dissolved in methanol and then

treated with diethyl ether and petroleum ether. A solid precipitated. It was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 13.7 min, 0-54 % linear gradient of acetontirile in water over 18 min) and lyophilized. Finally, it was dissolved in methanol, precipitated with ether, collected and dried. 45.5 mg were recovered, corresponding to a yield of 68 %. $^{1}$H NMR (DMSO-$d_6$): 6.56 (b, 6H, $NH_3$), 6.48 (s, 1H, DCA), 1.94 (s, 1H, OAc) ppm. $^{195}$Pt NMR (DMSO-$d_6$): 1227 ppm. Elemental analysis calculated for $C_4H_{10}Cl_4N_2O_4Pt$ (*3.5 $H_2O$): C, 8.73; H, 3.12; N, 5.09; %. Found: C, 8.21; H, 2.79; N, 5.28 %.

***ctc*-[Pt(NH$_3$)$_2$(Asp)(OAc)Cl$_2$] (CAAc).** Oxoplatin (46.3 mg, 0.14 mmol) and aspirin anhydride (71.0 mg, 0.21 mmol, 1.5 Eq) were stirred in 3 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in acetonitrile (3 mL) and acetic anhydride (2 mL) was added to the mixture. After 2 h at RT, the mixture turned to a bright yellow solution. It was filtered, concentrated and precipitate with a 1 : 1 mixture of diethyl ether and petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 16.0 min, 0-60 % linear gradient of acetontirile in water over 20 min) and then lyophiolized. Finally, it was dissolved in acetone and precipitated with ether, collected and dried. 23.7 mg were recovered, corresponding to a yield of 32 %. $^{1}$H NMR (DMSO-$d_6$): 7.86 (d, 1H, Asp), 7.53 (t, 1H, Asp), 7.31 (t, 1H, Asp), 7.09 (d, 1H, Asp), 6.60 (m, 6H, $NH_3$), 2.30 (s, 3H, Asp), 1.94 ppm (s, 3H, OAc). $^{195}$Pt NMR (DMSO-$d_6$): 1226 ppm. Elemental analysis calculated for $C_{11}H_{16}Cl_2N_2O_6Pt$ (*3$H_2O$): C, 22.31; H, 3.71; N, 4.73; %. Found: C, 22.45; H, 3.30; N, 4.53 %.

***ctc*-[Pt(NH$_3$)$_2$(Ibu)(OAc)Cl$_2$] (CIAc).** Oxoplatin (42.0 mg, 0.13 mmol) and ibuprofen anhydride (59.5 mg, 0.15 mmol, 1.2 Eq) were stirred in 4 mL of DMSO for 1 day at RT. The mixture was then filtered, recovering the mother liquor that was treated with ether, affording a two-phase system. The ether phase was removed after centrifugation. This procedure was repeated several times until a sticky yellow solid was obtained. It was suspended in DMF (1 mL) and acetic anhydride (1 mL) was added to the mixture. After 2 h at 60 °C, the mixture turned to a bright yellow solution. It was filtered, concentrated and precipitate with a 1 : 1 mixture of diethyl ether and petroleum ether. The solid was recovered by centrifugation, and the product was isolated on preparative HPLC (peak at 13.9 min, 10-100 % linear gradient of acetontirile in water over 15 min) and then lyophiolized. Finally, it was dissolved in acetone

and precipitated with ether, collected and dried. 42.4 mg were recovered, corresponding to a yield of 58 %. $^{1}$H NMR (DMSO-$d_6$): 7.22 (d, 2H, Ibu), 7.06 (d, 2H, Ibu), 6.53 (m, 6H, $NH_3$), 3.65 (q, 1H, Ibu), 2.41 (d, 2H, Ibu), 1.92 (s, 3H, OAc), 1.81 (m, 1H, Ibu), 1.30 (d, 3H, Ibu), 0.86 ppm (d, 6H, Ibu). $^{195}$Pt NMR (DMSO-$d_6$): 1226 ppm. Elemental analysis calculated for $C_{15}H_{26}Cl_2N_2O_4Pt$ (*1.5 $H_2O$): C, 30.46; H, 4.94; N, 4.74; %. Found: C, 30.30; H, 4.61; N, 4.87 %.

Stability studies

The eight triple action compounds were dissolved in ACN to obtain 5 mM solutions. 200 µL of this solution were diluted to 2.0 mL with phosphate buffer solution (pH = 7, [P] = 50 mM). These solutions were kept at 37 °C and monitored by HPLC.

**Biological methods**

Experiments with Cultured Human Cells.

Platinum(IV) complexes were dissolved in DMSO to stock solutions of 1 mg/mL just before the experiment, and a calculated amount of drug solution was added to the cell growth medium to a final solvent concentration of 0.5%, which had no discernible effect on cell killing. Cisplatin (CDDP) and oxaliplatin (OXP) were dissolved just before the experiment in a 0.9% NaCl solution. CDDP, OXP and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chemical Co, St.Louis, USA.

**Cell cultures.** Human pancreatic (PSN-1) and colon (HCT-15 and LoVo) carcinoma cells and non-transformed embryonic kidney cells (HEK293) were obtained from American Type Culture Collection (ATCC, Rockville, MD). The human thyroid carcinoma cell line (BCPAP) was provided by the Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany). The human ovarian cancer cell line 2008 and its cisplatin resistant variant, C13*, were kindly provided by Prof. G. Marverti (Dept. of Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy).

Cell lines were maintained in the logarithmic phase at 37°C in a 5% carbon dioxide atmosphere using the following culture media containing 10% fetal calf serum (Euroclone, Milan, Italy), antibiotics (50 units·mL$^{-1}$ penicillin and 50 µg·mL$^{-1}$ streptomycin) and 2 mM l-glutamine: i)

RPMI-1640 medium (Euroclone) for HCT-15, BCPAP, PSN-1, 2008 and C13*; ii) D-MEM medium (Euroclone) for HEK293 cells; iii) F-12 HAM's for LoVo cells.

Spheroid culturing

Spheroids were initiated in liquid overlay by seeding $2.5 \cdot 10^3$ PSN1 cells/well in phenol red-free RPMI-1640 medium (Sigma Chemical Co.), containing 10% FCS and supplemented with 20% methyl cellulose stock solution. A total of 150 µl of this cell suspension was transferred to each well of a round bottom non-tissue culture treated 96 well-plate (Greiner Bio-one, Kremsmünster, Austria) to allow spheroid formation within 72 h.

Cytotoxicity

**MTT assay.** The growth inhibitory effect towards human cell lines was evaluated by means of MTT (tetrazolium salt reduction) assay. Briefly, $3\text{-}8 \cdot 10^3$ cells/well, dependent upon the growth characteristics of the cell line, were seeded in 96-well microplates in growth medium (100 µL) and then incubated at 37°C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 10 µL of a 5 mg·mL-1 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution, and after 5 h additional incubation, 100 µL of a sodium dodecylsulfate (SDS) solution in HCl 0.01 M were added. After overnight incubation, the inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs drug concentration. $IC_{50}$ values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

**Acid phosphatase (APH) assay.** An APH modified assay was used for determining cell viability in spheroids. Briefly, the pre-seeded spheroids were treated with fresh medium containing the compound to be studied at the appropriate concentration. Triplicate cultures were established for each treatment. After 72 h, each well was treated with 100 µL of the assay buffer (0.1 M sodium acetate, 0.1% Triton-X-100, supplemented with ImmunoPure p-

nitrophenyl phosphate; Sigma Chemical Co.) and, following 3 h of incubation, 10 μL of 1 M NaOH solution were added. The inhibition of the cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 405 nm, using a Bio-Rad 680 microplate reader. Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance (T/C) and plotted vs drug concentration. $IC_{50}$ values, the drug concentrations that reduce the mean absorbance at 405 nm 50% of those in the untreated control wells, were calculated by four parameter logistic (4-PL) model. Evaluation was based on means from at least four independent experiments.

**Cellular uptake.** PSN1 cells ($2.5 \cdot 10^6$) were seeded in 75 $cm^2$ flasks in growth medium (20 mL). After overnight incubation, the medium was replaced and the cells were treated with tested compounds for 24 h. Cell monolayers were washed twice with cold PBS, harvested and counted. Samples were than subjected to three freezing/thawing cycles at -80 °C, and then vigorously vortexed. The samples were treated with highly pure nitric acid (Pt: $\leq 0.01$ $\mu g \cdot kg^{-1}$, TraceSELECT® Ultra, Sigma Chemical Co.) and transferred into a microwave teflon vessel. Subsequently, samples were submitted to standard procedures using a speed wave MWS-3 Berghof instrument (Eningen, Germany). After cooling, each mineralized sample was analyzed for platinum by using a Varian AA Duo graphite furnace atomic absorption spectrometer (Varian, Palo Alto, CA; USA) at the wavelength of 324.7 nm. The calibration curve was obtained using known concentrations of standard solutions purchased from Sigma Chemical Co. purchased from Sigma Chemical Co.

**DNA platination.** PSN1 cells ($3 \cdot 10^6$) were seeded in 10 cm Petri dishes in 10 mL of culture medium. Subsequently, cells were treated with tested complexes for 24 h. DNA was extracted and purified by a commercial spin column quantification kit (Qiagen DNeasy Blood and Tissue Kit). Only highly purified samples (A260/A230 □ 1.8 and A280/A260 □ 2.0) were included for analysis to avoid any artefacts. The samples were completely dried and re-dissolved in 200 μL of Milli-Q water (18.2 MΩ) for at least 20 min at 65 °C in a shaking thermo-mixer, mineralized and analysed for total Pt content by GF-AAS as described above.

**Histone deacetylase assay**. Histone deacetylase activity was determined using Fluor-de-Lys® HDAC fluorometric activity assay kit (Enzo Life Sciences International, Inc., Plymount Meeting, PA, U.S.A.). PSN1 cells ($5 \cdot 10^4$ seeded in 96-well microplates) were treated for 24 h with tested complexes, and then processed as reported by the manufacturer's instructions.

Fluorescence was measured using a Fluoroskan Ascent FL (Labsystem, Finland) plate reader, with excitation at 360 nm and emission at 460 nm.

**COX-2 inhibition.** The inhibition of COX-2 was measured in PSN-1 cells by using COX activity assay kit (Cayman Chemical, Ann Arbor, USA). Cells were incubated for 24 h with 1 μM of tested complexes and then processed as reported by the manufacturers' instructions. The inhibition of the COX-2 induced by the tested complexes was detected by measuring the absorbance of each well at 590 nm, using a Bio-Rad 680 microplate reader.

**ROS production.** The production of ROS was measured in PSN-1 and HEK293 cells ($10^4$ per well) grown for 24 h in 96-well plates in complete medium without phenol red (Sigma Chemical Co.). Cells were then washed with PBS and loaded with 10 μ M 5-(and-6)-chloromethyl-2′ ,7′ -dichlorodihydrofluorescein diacetate, acetyl ester (CM–$H_2$DCFDA) (Molecular Probes-Invitrogen) for 25 min in the dark. Afterwards, cells were washed with PBS and incubated with increasing concentrations of the tested complexes. The fluorescence increase was estimated with a plate reader (Fluoroskan Ascent FL, Labsystem, Finland) at 485 (excitation) and 527 nm (emission). Antimycin (3 μM, Sigma Chemical Co.), a potent inhibitor of Complex III in the electron transport chain, was used as positive control.

**Mitochondrial membrane potential (ΔΨ).** ΔΨ was assayed using the Mito-ID Membrane Potential Kit according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY). Briefly, PSN-1 and HEK293 cells were seeded onto 96-well microplates at $5 \cdot 10^4$ cells/well. After 24 h, cells were treated with tested compounds for 12 h. An equal volume of cationic dye loading solution was added to each well and cell plates were incubated for additional 30 min at 37°C. Plates were read at excitation/emission wavelengths 490 and 590 nm using a fluorescence microplate reader (Fluoroskan Ascent FL, Labsystem, Finland).

## Compounds characterization

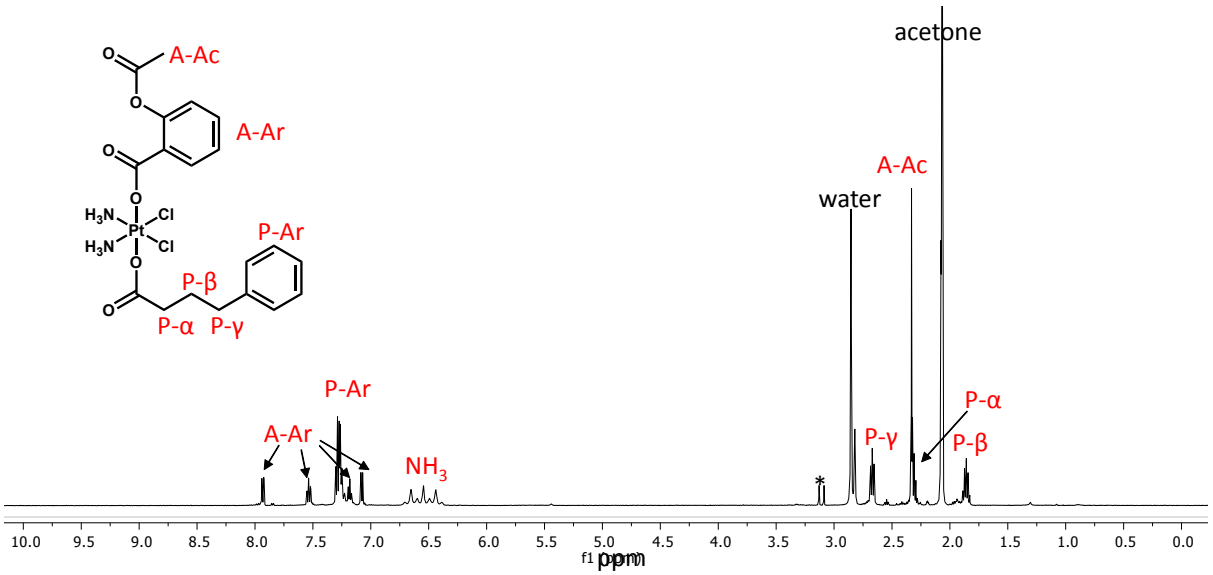


**Figure S1.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(PhB)(Asp)Cl$_2$] (CPA)** in acetone-$d_6$. * indicates an unidentified impurity present in the sample.

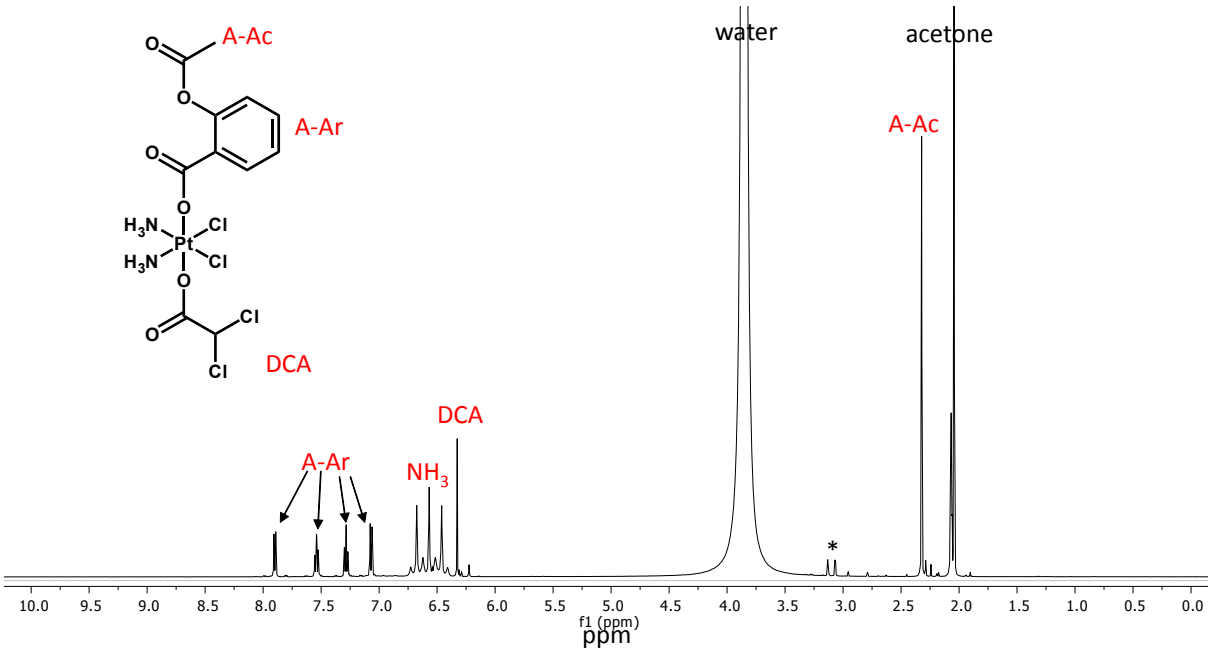


**Figure S2.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Asp)(DCA)Cl$_2$] (CAD)** in acetone-$d_6$. * indicates an unidentified impurity present in the sample.

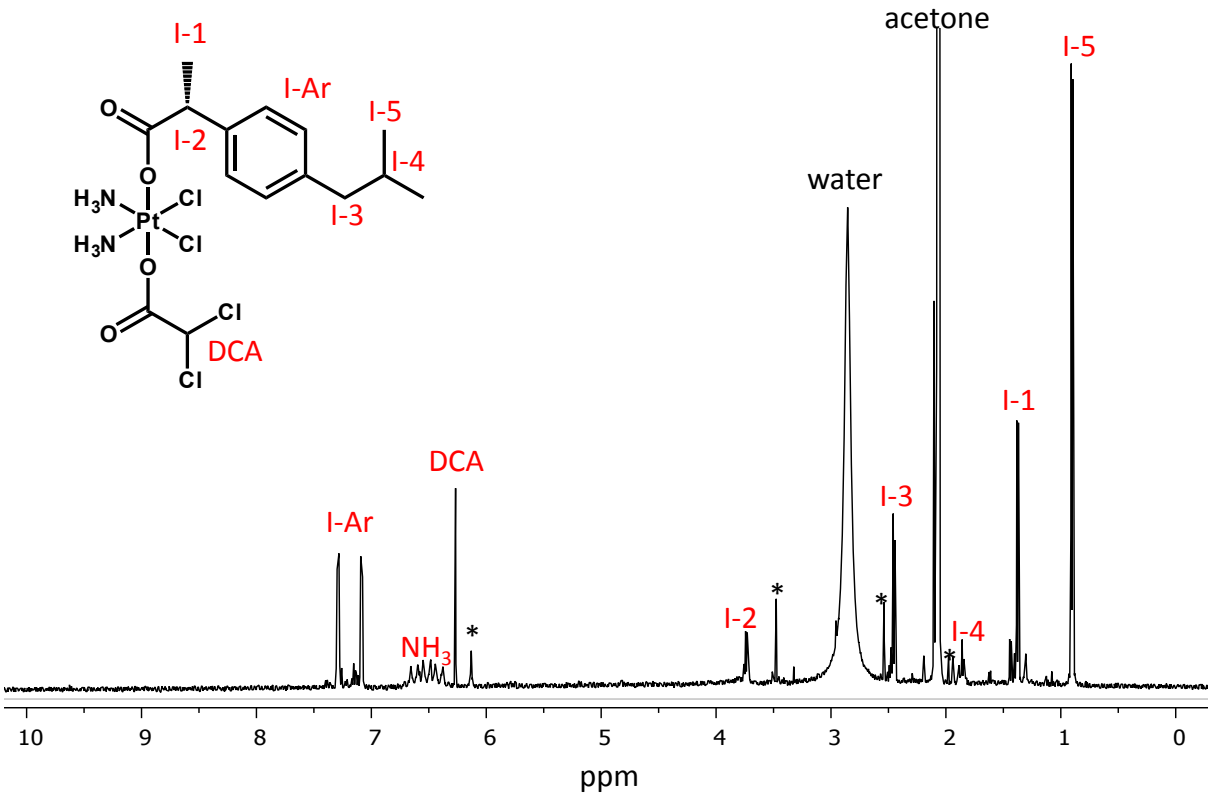


**Figure S3.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Ibu)(DCA)Cl$_2$] (CID)** in acetone-$d_6$. * indicates impurities present in the NMR sample.

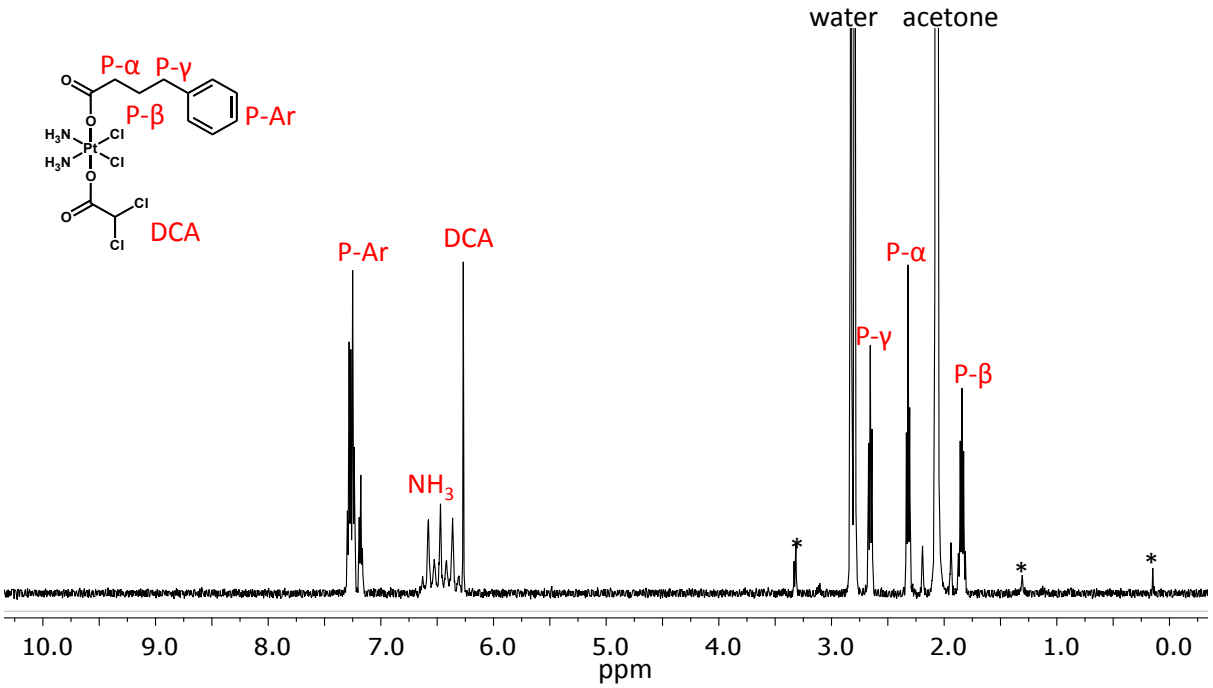


**Figure S4.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(PhB)(DCA)Cl$_2$] (CPD)** in acetone-$d_6$. * indicates unidentified impurities present in the NMR sample.

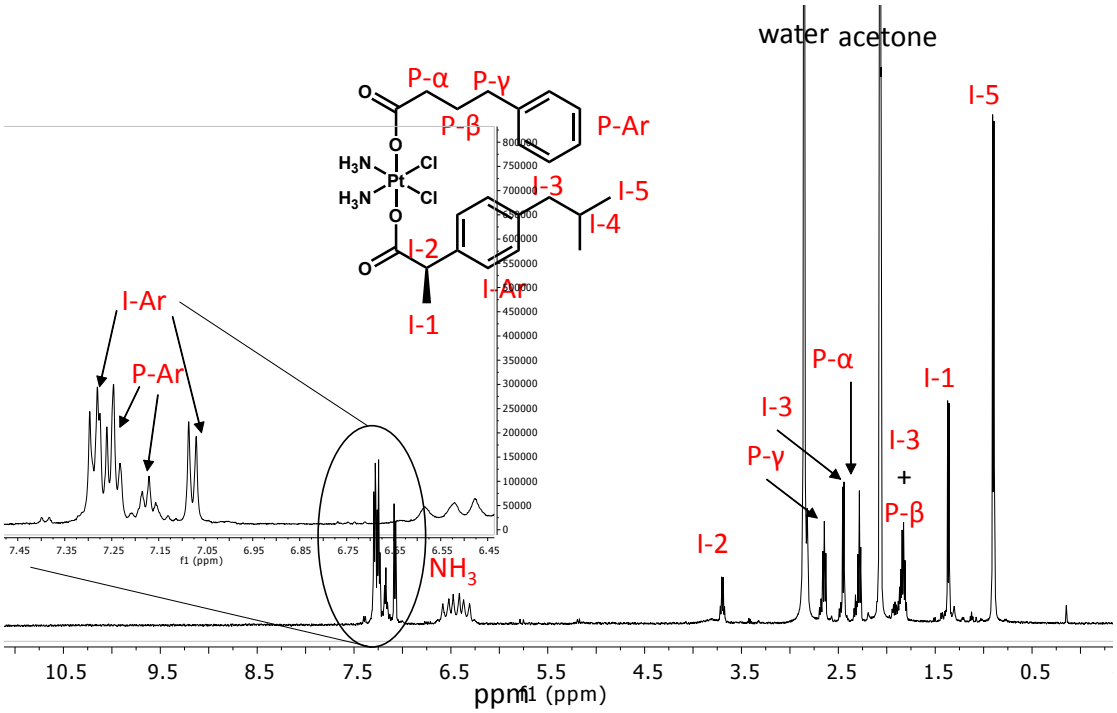


**Figure S5.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(PhB)(Ibu)Cl$_2$] (CPI)** in acetone-$d_6$.

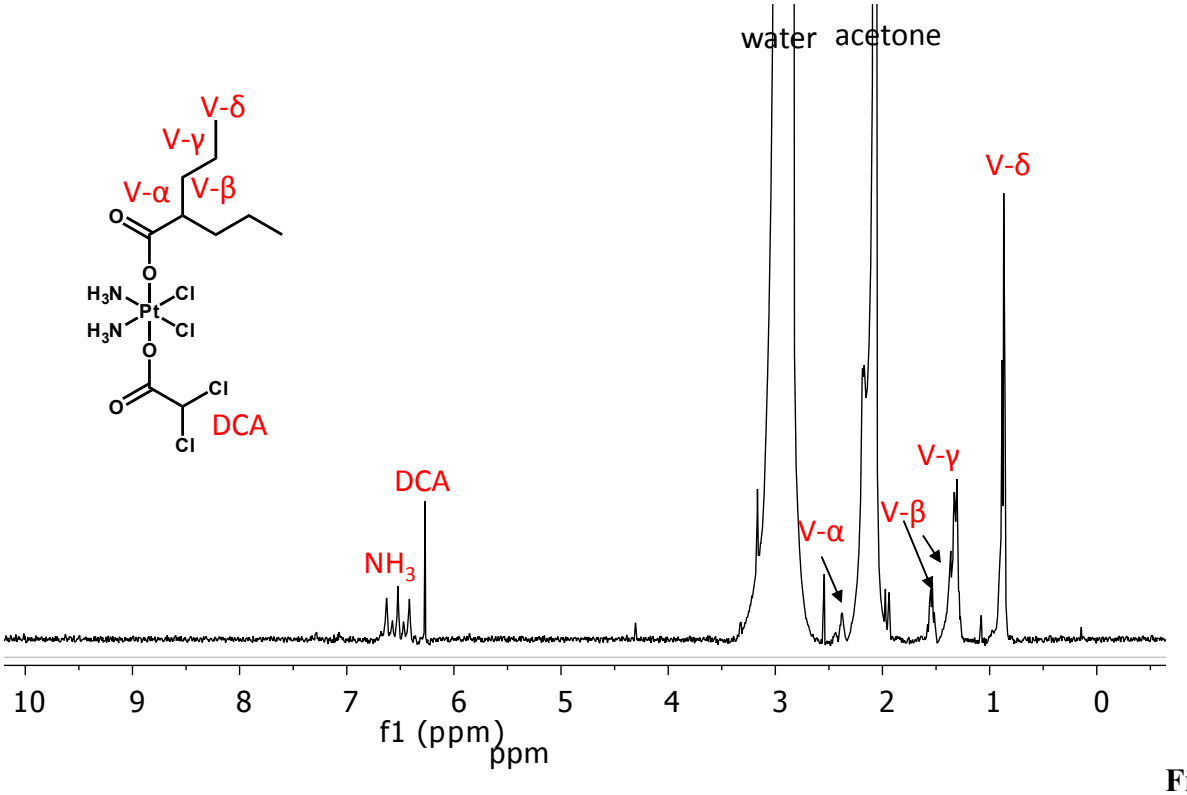


**Figure S6.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Val)(DCA)Cl$_2$] (CVD)** in acetone-$d_6$.

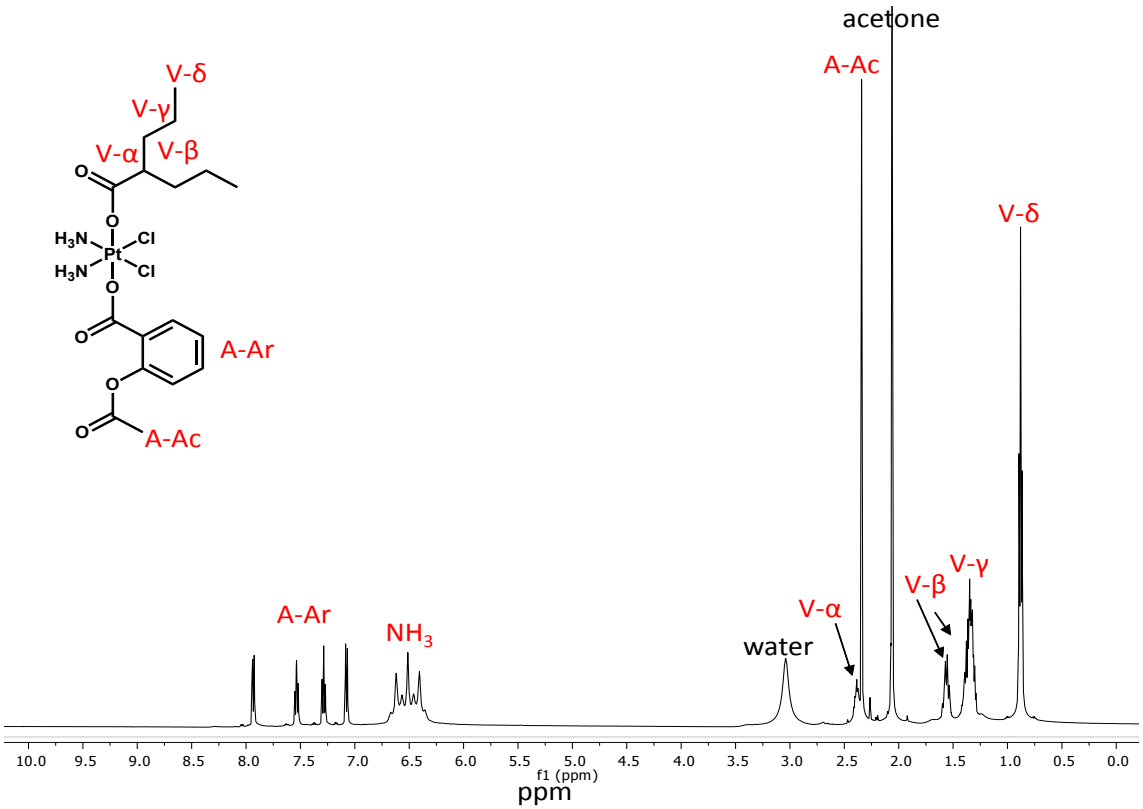


**Figure S7.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Val)(Asp)Cl$_2$] (CVA)** in acetone-$d_6$.

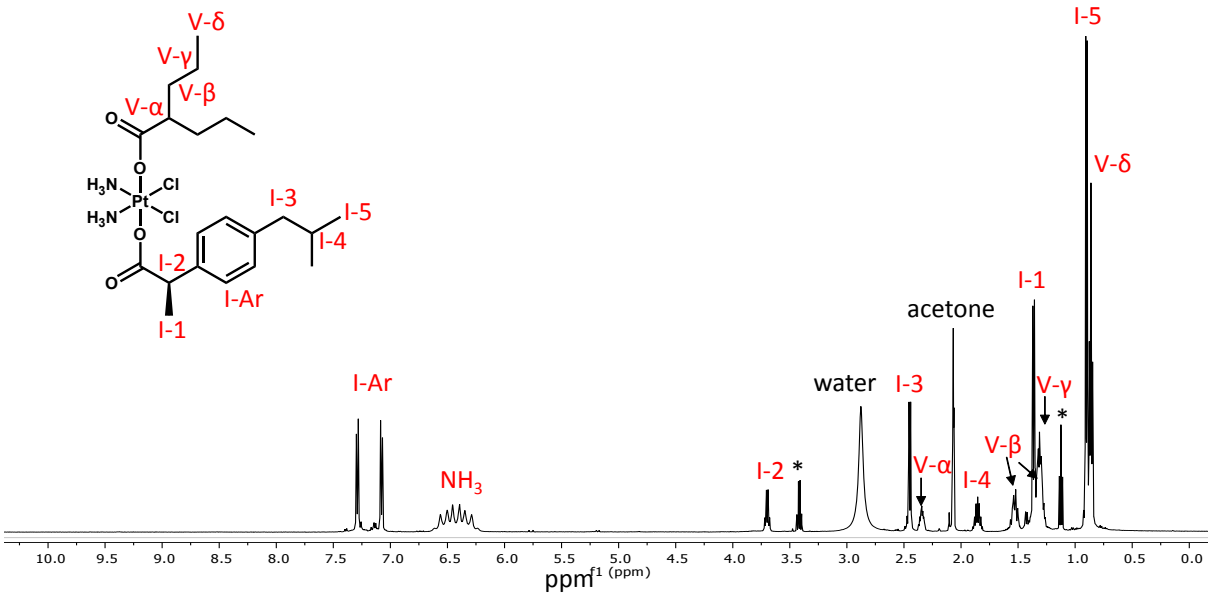


**Figure S8.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Val)(Ibu)Cl$_2$] (CVI)** in acetone-$d_6$. * indicates impurities present in the NMR sample.

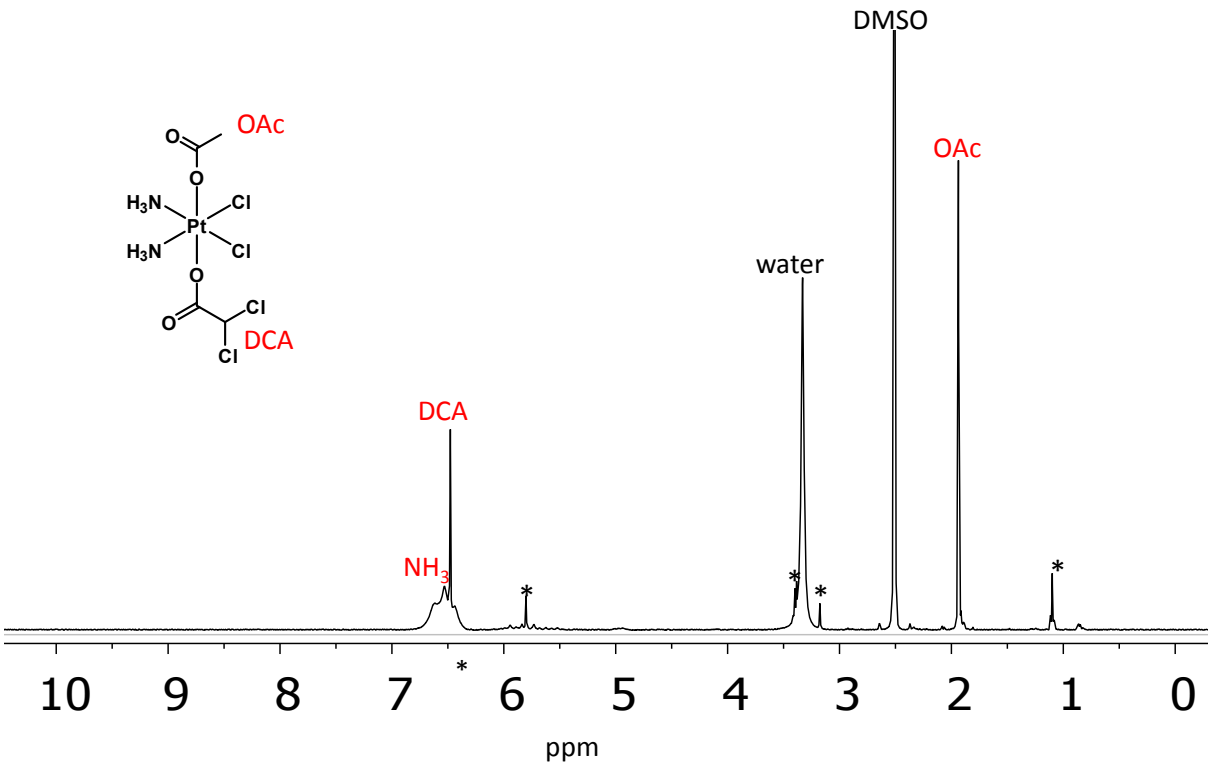


**Figure S9.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(DCA)(OAc)Cl$_2$] (CDAc)** in DMSO-$d_6$. * indicates impurities present in the NMR sample.

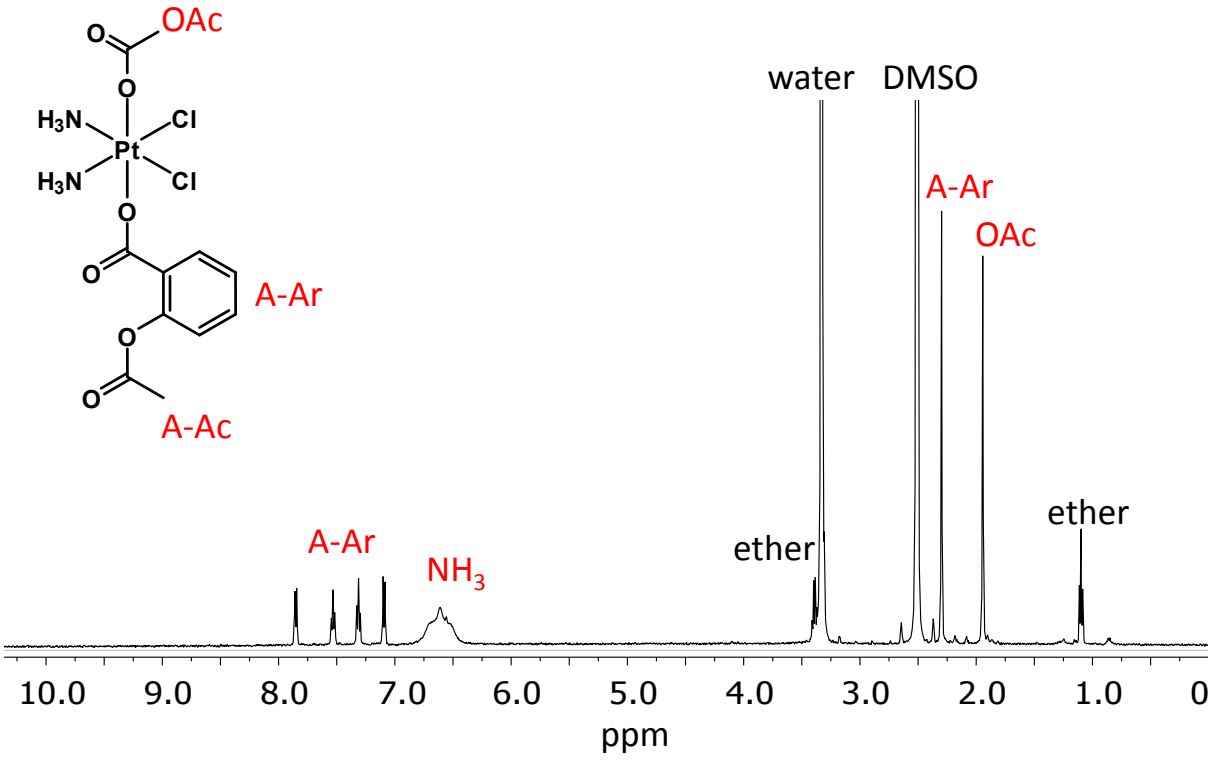


**Figure S10.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Asp)(OAc)Cl$_2$] (CAAc)** in DMSO-$d_6$.

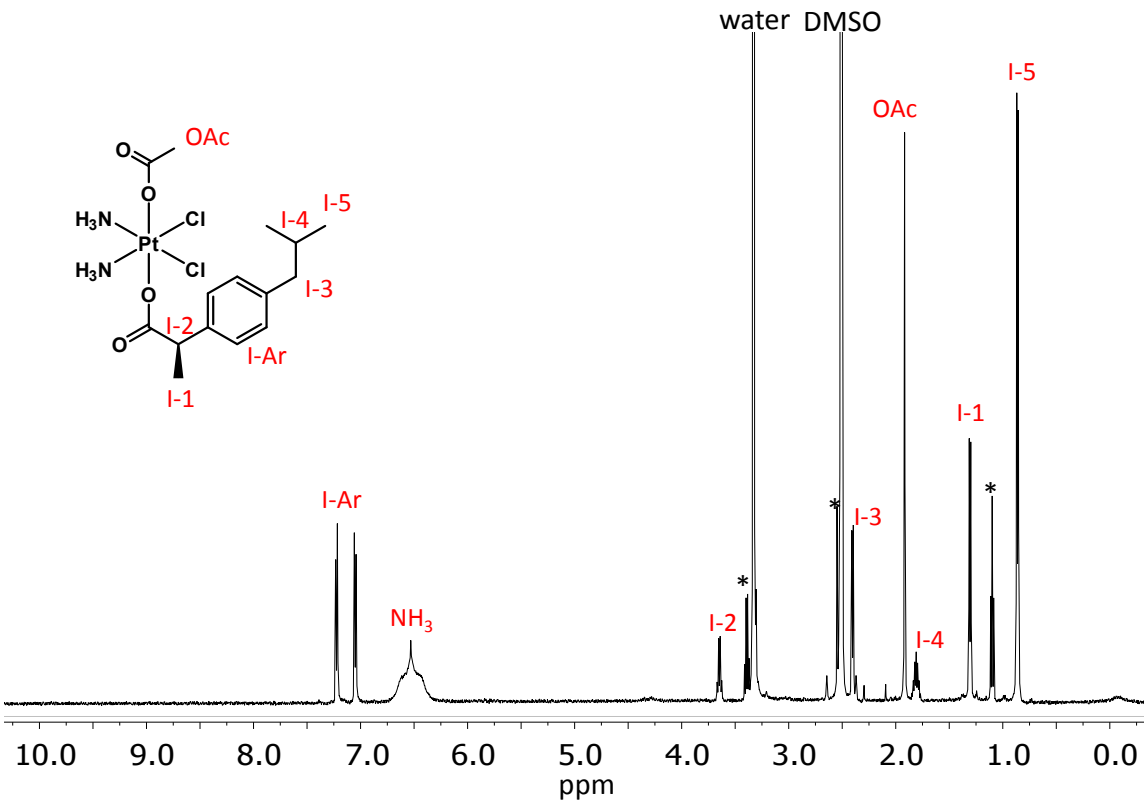


**Figure S11.** $^{1}$H NMR spectrum of ***ctc*-[Pt(NH$_3$)$_2$(Ibu)(OAc)Cl$_2$] (CIAc)** in DMSO-$d_6$.

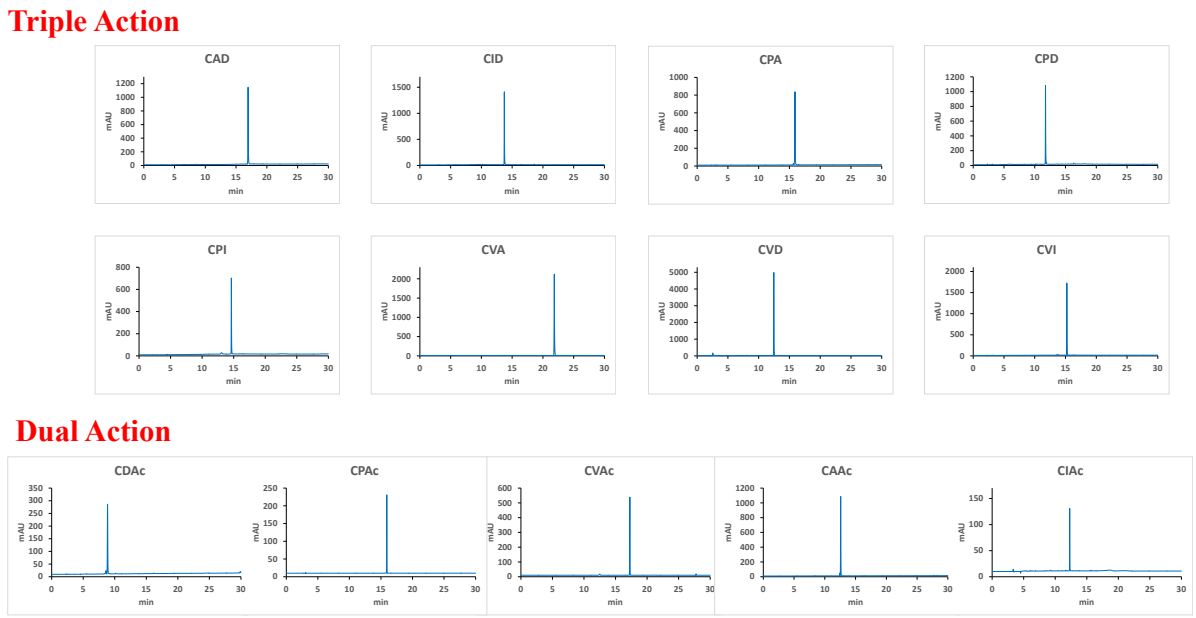


**Figure S12.** HPLC chromatograms of triple and dual action compounds.

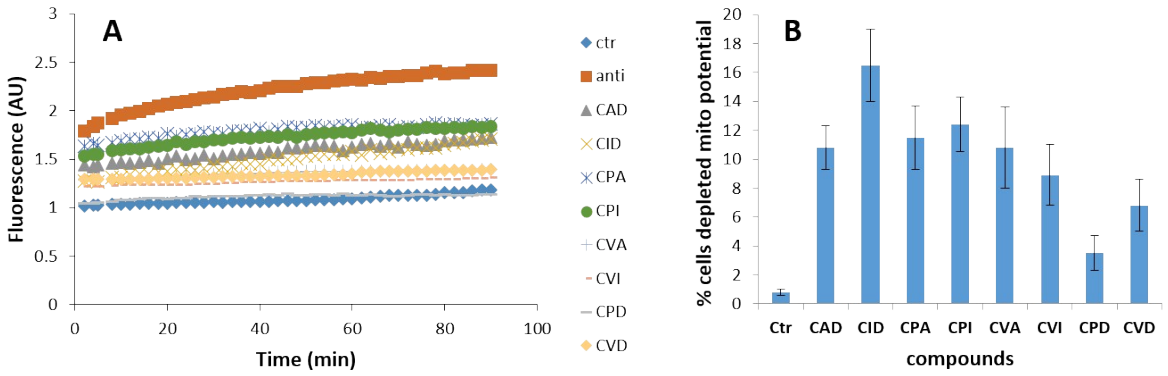


**Figure S13** - A) ROS production. HEK293 cells were preincubated in PBS/10 mM glucose medium for 20 min at 37 °C in the presence of 10 µM CM-DCFDA and then treated with equimolar doses (10 µM) of Pt(IV) derivatives. The fluorescence of DCF was measured. Data are the means of three independent experiments. B) Effects on cellular mitochondrial membrane potential. HEK293 cells were treated for 24 h with 1 µM of tested compounds. The percentage of cells with hypopolarized mitochondrial membrane potential was determined by Mito-ID® Membrane Potential Kit.

**Table S1.** Retention time on analytical HPLC.

| **Monocarboxylated** | |
|---|---|
| **Compound** | **Min** |
| **CAOH** | 10.8[1] |
| **CIOH** | 11.2[2] |
| **CPOH** | 14.0[1] / 9.0[2] |
| **CVOH** | 15.0[1] / 9.4[2] |

| **dual action** | |
|---|---|
| **compound** | **min** |
| **CDAc** | 8.8[1] |
| **CPAc** | 15.9[1] |
| **CVAc** | 17.3[1] |
| **CAAc** | 12.6[1] |
| **CIAc** | 12.3[2] |

| **triple action** | |
|---|---|
| **compound** | **Min** |
| **CAD** | 16.9[1] |
| **CPA** | 20.5[1] |
| **CID** | 16.9[2] |
| **CPD** | 19.6[1] / 11.8[2] |
| **CPI** | 26.0[1] / 14.6[2] |
| **CVD** | 19.6[1] / 12.5[2] |
| **CVA** | 21.9[1] |
| **CVI** | 15.2[2] |

[1] 0 – 90 % ACN linear gradient in water over 30 min.

[2] 0 – 100 % ACN linear gradient in water over 15 min followed by 100 % ACN for 15 min.

**Table S2.** Cytotoxicity assessed by the MTT test of dual action compounds. Cells (3-8×$10^4$ $mL^{-1}$) were treated for 72 h with increasing concentrations of tested compounds. $IC_{50}$ values were calculated by a four parameter logistic model ($P < 0.05$). Resistant factor (RF) is defined as $IC_{50}$ resistant/parent line.

| | $IC_{50}$(μM)±S.D. | | | | | |
|---|---|---|---|---|---|---|
| **Compound** | **HCT-15** | **BCPAP** | **PSN-1** | **2008** | **C13*** | **LoVo** |
| **CAAc** | 4.53±1.2 | 0.31±0.1 | 0.25±0.1 | 0.65±0.1 | 0.71±0.1 | 1.11±0.7 |
| **CDAc** | 4.05±1.1 | 0.73±0.2 | 0.94±0.3 | 0.86±0.2 | 0.94±0.2 | 1.27±0.6 |
| **CIAc** | 1.98±0.8 | 1.98±0.8 | 0.99±0.6 | 0.98±0.1 | 1.50±0.3 | 1.25±0.5 |
| **CPAc** | 4.52±1.2 | 2.25±0.7 | 1.58±0.6 | 0.78±0.2 | 1.02±0.7 | 4.02±1.1 |
| **CVAc** | 5.39±1.5 | 3.06±1.2 | 2.61±0.8 | 1.59±0.6 | 1.75±0.7 | 5.42±0.6 |

**Table S3.** Cytotoxicity assessed by the MTT test in colon oxaliplatin sensitive (LoVo) and resistant (LoVo OXP) cancer cell lines, using oxaliplatin as reference. S.D. = standard deviation. Cells (3-8 × 104 mL−1) were treated for 72 h with increasing concentrations of tested compounds. The cytotoxicity was assessed by the MTT test. IC50 values were calculated by a four parameter logistic model ($P < 0.05$). Resistant factor (RF) is defined as $IC_{50}$ resistant/parent line

| **Class** | **Compound** | **$IC_{50}$ (μM)± S.D.** | | |
|---|---|---|---|---|
| | | **LoVo** | **LoVo OXP** | **RF** |
| **COXi/PDKi** | **CAD** | 0.755±0.06 | >3.125 | >4.14 |
| | **CID** | 0.285±0.02 | >3.125 | >10.9 |
| **HDACi/COXi** | **CPA** | 0.055±0.01 | 0.09±0.23 | 1.63 |
| | **CPI** | 0.211±0.08 | >3.125 | >14.81 |
| | **CVA** | 0.97±0.08 | 1.69±0.12 | 1.74 |
| | **CVI** | 0.034±0.03 | 0.84±0.02 | 24.7 |
| **HDACi/PDKi** | **CPD** | 0.019±0.01 | 0.037±0.02 | 1.95 |
| | **CVD** | 0.757±0.23 | 1.14±0.01 | 1.51 |
| **reference** | **oxaliplatin** | 1.20±0.13 | 21.92±1.86 | 18.27 |

**Table S4** Cytotoxicity assessed by the MTT test of co-treatment of cancer cells with cisplatin, PhB and DCA alone or at 1:1:1 ratio. Cells ($5\times10^4$ $mL^{-1}$) were treated for 72 h with tested compounds. $IC_{50}$ values were calculated by a four parameter logistic model ($P < 0.05$).

| | $IC_{50}$ (μM) | | | | |
|---|---|---|---|---|---|
| **cmpd** | **HCT-15 colon** | **BCPAP thyroid** | **PSN-1 pancreas** | **LoVo colon** | **2008 ovary** |
| **1:1:1 cisplatin PhB, DCA** | 14.34 ±2.23 | 8.12 ±1.08 | 17.11 ±4.23 | 9.46 ± 2.33 | 1.98 ±0.45 |
| **CPD** | 1.06 ±0.13 | 0.11 ±0.03 | 0.10 ±0.02 | 0.019 ±0.01 | 0.33 ±0.04 |
| **cisPt** | 15.28 ±2.63 | 7.38 ±1.53 | 18.25 ±3.11 | 9.15 ± 2.07 | 2.22 ±1.02 |
| **PhB** | >50 | >50 | >50 | >50 | >50 |
| **DCA** | >50 | >50 | >50 | >50 | >50 |