Supporting Information

Alternative mechanism of action of DNP PtIV prodrug: intracellular cisplatin release and mitochondria-mediated apoptotic pathway

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Experimental Procedures

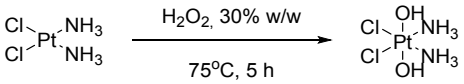
Materials: Cisplatin, N,N'-Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), hexafluorophosphate (HBTU), dimethylaminopyridine (DMAP), H_2O_2 37% w/w were purchased from commercial sources (Aldrich, Alfa, TCI, etc.), and used without further purification. Naproxen was isolated from the pounded tablets by acidification of the suspension and extraction with methylene chloride. Hydrochloric acid, N,N-dimethylformadide (DMF), toluene, MeOH, EtOH, CH_2Cl_2, DMEM, PBS, TEA and PI were purchased in Sigma Aldrich (USA), dimethylsylfoxide (DMSO) was purchased from VMR (Life Science). Flash column chromatography (300–400 mesh silica gel) was performed for compound purification. Analytical thin-layer chromatography (TCL) was performed on Merck silica gel aluminum plates with F-254 indicator. Compounds were visualized by irradiation with UV light or iodine staining. For mitochondrial potential measurements JC-1 dye (Mitochondrial Membrane Potential Probe) (Invitrogen, USA) was utilized, the staining was performed according to the manufacturer's protocol.

Instrumentation: ^{1}H, ^{13}C and ^{195}Pt NMR spectra were recorded on a Brucker-Avance instrument at 400 MHz (^{1}H), 101 MHz (^{13}C) and 86 MHz (^{195}Pt). Deuterochloroform ($CDCl_3$), deuteromethanol (CD_3OD) and dimethylsulfoxide-d6 (DMSO-d6) were used solvents. Chemical shifts are given in ppm

on the δ scale relative to hexamethyldisiloxane as an internal standard for ^{1}H, ^{13}C NMR, $K_2[PtCl_6]$ solution for ^{195}Pt NMR. The amount of drug required was 4 mg for ^{1}H and 15 mg for ^{13}C and ^{195}Pt NMR. The signal decay time was 2 minutes for ^{1}H, 1 hour for ^{13}C and 8 hours for ^{195}Pt NMR spectra. High-resolution mass spectra were recorded on an Orbitrap Elite mass spectrometer (Thermo Scientific). IR spectra were obtained on ThermoNicolet ISFT-IR (USA). Liquid chromatography–mass spectrometry was performed by a Shimadzu Prominence LC-20 system with a column oven and fraction collector coupled to a single quadrupole mass spectrometer Shimadzu LCMS-2020 with a dual DUIS-ESIAPCI ionization source. XANES spectra were measured at the L3 edge of platinum at the STM station of the Kurchatov Center of Synchrotron Radiation. The storage ring of the synchrotron is powered by an energy of 2.5 GeV, the average current in the ring is about 80 mA. P content was determined by inductively coupled plasma mass spectrometry using an ICP-MS 7500a instrument (Agilent Technologies). The device was set up to work with solutions that have a low total concentration of solutes (RF Power 1500Vt, Carrier Gas Flow Rate 1.10 l/min, Sample Flow Rate 0.4 ml/min, Sampling Depth 4mm, t°C, 2°C). Flow cytometry data were obtained using a NovoCyte 2000R flow cytometer (ACEA Biosciences, USA), and the obtained data were analyzed using NovoExpress v.1.2.4 software.

Synthetic procedures

Synthesis of 1 (Oxoplatin)

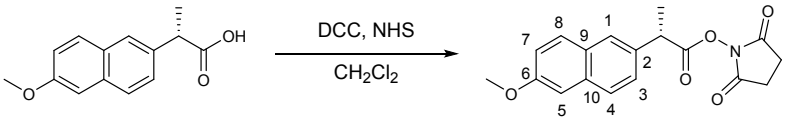


1, 92%

To 500 mg (1.67 mmol) of cisplatin, 30 ml of 30% hydrogen peroxide solution were added dropwise. The mixture was stirred at 75°C for 5 hours. The solution was left overnight at room temperature to form a yellowish precipitate. The precipitate formed was separated, the solvent was removed under reduced pressure, the residue was collected with a small amount of water and washed with water, methanol, and diethyl ether, and 515 mg (92%) oxoplatin **1** was obtained.

IR: 3459 (OH), 3149, 1583 (c), 1440 (c), 1378 (s), 1074 (s, Pt-OH), 860 (s), 574 (br. s).

Synthesis of 2 (Naproxen NHS ester)

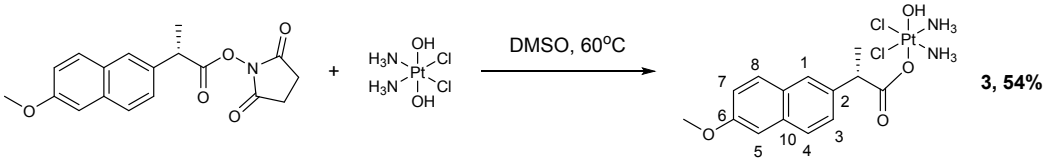


2, 81%

The mixture of 500 mg (2.17 mmol) of naproxen, 250 mg of NHS (2.17 mmol) and 492 mg of DCC (2.39 mmol) was dissolved in 20 ml of DCM and stirred for 2 hours. The dicyclohexylurea precipitate was separated by filtration, the solution was evaporated under reduced pressure. The residue was suspended in ethyl acetate, dicyclohexylurea was filtrated again. The resulted solution was evaporated under reduced pressure. The crude product was purified by flash-chromatography with DCM as eluent. 575 mg (81%) of naproxen NHS ester **2** was obtained as white powder.

^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.83 - 7.78 (m, 3H, Ph-**H1**, Ph-**H4**, Ph-**H8**), 7.46 – 7.44 (d, *J*=8.44 Hz, 1H Ph-**H3**), 7.31 (s, 1H, Ph-**H5**), 7.18 – 7.15 (dd, *J1*=8.99, *J2*=2.45 Hz, 1H, Ph-**H7**), 4.43 - 4.34 (q, 1H *J*=7.09 Hz, $C(CH_3)$-***H***) 3.86 (s, 1H, OCH_3) 2.77 (s, 4H, CH_2CH_2), 1.59 - 1.57 (d, *J*=7.09 Hz, 3H, C-C**H_3**).

Synthesis of 3 (NP)



3, 54%

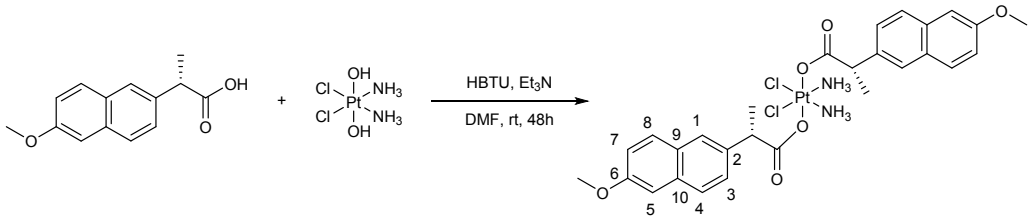
62 mg (0.18 mmol) of oxoplatin **1** and 73 mg (0.22 mmol) of naproxen NHS ester **2** were dissolved in 4.65 ml of DMSO and the reaction was stirred at 50°C overnight. The remained solid was separated by centrifugation, the solution was quenched by 45 ml of ether. After 10-15 minutes, the brightly colored DMSO layer was separated and the procedure was repeated until sticky beige solid was obtained. 1 ml of methanol was added, and the mixture was suspended. The suspension was centrifugated, the solid was washed with ether and dried on air. The solution was quenched with excess of ether and centrifugated. The solid was washed with ether and air dried.

An additional amount of **3** (NP) was obtained by evaporating the combined liquid fractions under reduced pressure. A few drops of methanol were added to the residue, the mixture was sonificated, 5 ml of ether were added, and the resulting suspension was centrifuged. 55 mg (54%) of **NP** (**3)** was obtained as beige powder.

^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.74 - 7.68 (m, 3H, Ph-**H1**, Ph-**H4**, Ph-**H8**), 7.48 – 7.45 (dd, *J1*=8.50, *J2*=1.71 Hz, 1H Ph-**H3**), 7.25 (s, 1H, Ph-**H5**), 7.12 – 7.10 (dd, *J1*=8.96, *J2*=2.54 Hz, 1H, Ph-**H7**), 6.09 - 5.83 (br. tr., 6H, N**H_3**,), 3.84 (s, 3H, OC**H_3**) 3.74 - 3.72 (q, *J*=7.15 Hz, 1H, $C(CH_3)$-***H***) 1.38 - 1.36 (d, *J*=7.09 Hz, 3H, C-C**H_3**).

HRMS: calculated $[C_{14}H_{20}Cl_2N_2O_4Pt+H]^+$ 546.0521 found $[C_{14}H_{20}Cl_2N_2O_4Pt+H]^+$ 546.0531.

Synthesis of 4 (DNP)



40 mg (0.12 mmol) of oxoplatin **1**, 82 mg (0.36 mmol) of naproxen and 136 mg of HBTU (0.36 mmol) were dissolved in 4 ml of anhydrous DMF. 50 µl (0.36 mmol) of TEA were added and the solution stirred at room temperature for 48 hours. The solution was concentrated under reduced pressure to the volume of 1 ml. The solution was added dropwise to 10 ml of diethyl ether and the suspension was stirred overnight. The precipitate was separated by centrifugation, washed with methanol and ether and air dried. 48 mg (52%) of **DNP (4)** were obtained as yellow powder.

^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 7.76 - 7.70 (m, 6H Ph-**H1**, Ph-**H4**, Ph-**H8**) 7.48 – 7.46 (dd, *J1*=8.62, *J2*=1.1 Hz, 2H, Ph-**H3**) 7.27 (s, 2H, Ph-**H5**) 7.14 - 7.11 (dd, *J1*=8.93, *J2*=2.26 Hz, 3H, Ph-**H7**), 6.76 - 6.35 (br. s., 6H, N**H$_3$**), 3.85 - 3.80 (m, 8H, OC**H$_3$**, C(CH$_3$)-**H**) 1.41 - 1.39 (m, 6H C-C**H$_3$**).

^{13}C NMR (101 MHz, DMSO-d$_6$, δ, ppm): 182.00, 156.92, 137.37, 133.04, 129.09, 128.36, 127.01, 126.36, 125.50, 118.38, 105.65, 55.14, 19.84.

^{195}Pt NMR (86 MHz, DMSO-d_6, δ, ppm): 1216.95

HRMS: calculated $[C_{28}H_{32}Cl_2N_2O_6Pt+H]^+$ 758.1358 found $[C_{28}H_{32}Cl_2N_2O_6Pt+H]^+$ 758.1361

MTT assay. The cytotoxicity of substances was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay. For this, 5,000 MCF-7 cells were seeded in 96-well plate in 100 µL of full DMEM (Gibco, USA) per well and incubated overnight. Cisplatin and **DNP** were dissolved in DMSO to 10 mM and then diluted with full DMEM to reach 50 nM -100 µM concentrations (final DMSO concentration in the media was less than 1%) and transferred to cells for 72 hours. The MTT reagent (Paneco LLC, Russia) then was added to cells up to final concentration of 0.5 g/l and incubated for 2 hours at 37ºC at 5% CO_2. The MTT solution was then discarded and 100 µl of DMSO (PharmaMed LLC, Russia) was added. The plates were swayed on a shaker (60 rpm) to solubilize the formazan. The absorbance was measured using a microplate reader (VICTOR X5 Light Plate Reader, PerkinElmer, USA) at a wavelength of 565 nm (in order to measure formazan concentration). The results were used to construct a dose-response graph and to estimate IC_{50} value (GraphPad Software, Inc., San Diego, CA).

XANES study of drug release in A549 tumor cells (Cell preparation)

Human lung carcinoma A549 cells were seeded in T25 culture flasks (700000 cells per flask). After 40 hours incubation and reaching 80% confluence, 10 µM of **DNP** and NP was added for 3 hours or 24 hours. Following the incubation, the drug-containing medium was removed, and the cells were washed thoroughly with ice cold PBS solution (pH 7.5, SIGMA) to remove residual medium. The cells were trypsinised and centrifuged at 2500 rpm for 3 min, the supernatant was removed, and the cells were resuspended in a solution of ammonium acetate (100 mM, 5 mL) and centrifuged again. Finally, the cell pellets were washed with a 70% ethanol solution, yielding a dry cell pellet. The samples were immediately freeze-dried for 24 hours and stored in a desiccator until they were analyzed. At the time of analysis, the freeze-dried cells were packed into a polycarbonate sample holder, and the open face secured with a Kapton tape (Kapton) window for XANES analysis. XANES spectra of L3 edge of platinum were recorded in the fluorescence signal detection mode.

Cellular platinum accumulation and distribution. Human carcinoma MCF-7 cells were seeded on the T-25 (Eppendorf) cell culture flasks and cultured to 80% confluence in full DMEM. Cisplatin and **DNP** were dissolved in DMSO to 10 mM concentration and then were diluted with full DMEM to reach 10 µM concentration. Cells were incubated with 4 ml of the prepared solutions for 3.5 hours in CO_2 incubator at 37ºC. Then the medium was decanted, the cells washed with PBS (10 mM, pH 7.4) two times, and collected by trypsinization. Then cell suspension was centrifuged at 2500g for 5 minutes in a Beckman centrifuge. The collected cells were resuspended in 1 ml PBS and counted using a TC20 automated cell counter (Biorad). Cell samples were divided into fractions by the differential centrifugation method.

Pellets of the MCF-7 cells were subjected to three freezing/thawing cycles at -80ºC, treated with 200 µl of PBS supplemented with 0.1 M NaOH and 0.05% Tween-20, and vigorously vortexed to lyse the cells. The homogenate was centrifuged at 600 g for 15 minutes at 4ºC to isolate the nuclear fraction. Then, the supernatant was carefully transferred to a new tube and centrifuged at 15000 g for 15 minutes at 4ºC to isolate the mitochondria fraction. The supernatant was a cytosol fraction, which was carefully transferred to another tube. All three fractions were treated with highly pure nitric acid (100 µl for nuclear and mitochondria fractions, and 300 µl for cytosol fraction) for at least 24 hours at room temperature and then for 2 hours at 65°C until complete dissolution.

Cytotoxicity in tumor spheroids (MTS assay). Tumor spheroid formation was performed using liquid overlay technique using agarose-coated plates as it was described earlier.[1,2] Briefly, 1.5% wt of agarose in PBS (pH 7.4) was heated on water bath for 15 min. Then, 50 µL of melted agarose was added to each well of a flat-bottom 96-well plate under sterile conditions. Plates with agarose were cooled down to room temperature for 15 minutes. Cells were seeded on agarose-coated plates (10,000 cells/well, 100 µL of media per each well) and incubated at 37°C and 5% CO_2 for 72 hours to generate spheroids. The formation of tumor spheroids was observed using the inverted light microscope. Then the solutions (0.1-20 µM) of CDDP and **DNP** were added to spheroids for 72 hours. The cytotoxicity was evaluated using colorimetric MTS assay based on the NAD(P)H-dependent dehydrogenase activity resulted in the reduction of MTS tetrazolium compound and generation the soluble formazan product in cell culture media. The formazan formation was measured at 490 nm using Varioskan Flash reader (Thermo Scientific, USA). The IC_{50} values were estimated using the GraphPad Prism Software.

Cell Cycle Analysis. MCF-7 cells (5×10^5) were seeded on a 6-well plate and incubated overnight. The cells were then treated with 0.2 µM and 1 µM of **DNP** and CDDP for 48 hours. Then the cells were detached with trypsin-EDTA, washed with PBS (pH 7.4), and resuspended in 1 ml of 50 µg/ml PI solution in the staining buffer (Becton Dickinson, Franklin Lakes, NJ, USA) for 15 minutes at room temperature in the dark. The PI fluorescence was measured on a NovoCyte 2000R flow cytometer (ACEA Biosciences, USA), and the cell cycle distribution was analyzed using NovoExpress v.1.2.4 software. A minimum 50,000 cells per sample was acquired and analyzed, setting the forward scatter (FSC) and side scatter (SSC) on the dot plot to differentiate the population of cells from cellular debris (FSC-A vs. SSC-A) and doublets (FSC-H vs. FSC-W).

Membrane potential measurement (JC-1 assay). MCF-7 cells (5×10^5) were seeded on a 6-well culture plate and incubated overnight. CDDP and **DNP** were added to the cells at 1 µM concentrations for 48 hours. After that, cells were harvested and resuspended in 1 mL warm phosphate-buffered saline (pH 7.4). JC-1 reagent was added to the final concentration of 2 µM for 30 minutes at 37°C and 5% CO_2. Then the cells were washed once by adding 2 mL of PBS (pH 7.4) and pelleted by centrifugation. Finally, the cells were resuspended in 300 µL PBS and analyzed using a flow cytometer NovoCyte 2000R (ACEA Biosciences) with 488 nm excitation using emission filters appropriate for Invitrogen Alexa Fluor 488 dye and R-phycoerythrin. Median fluorescence was estimated for each emission channel, and red/green fluorescence ratio was calculated.

Statistical Analysis

All the results were expressed as Mean ± Standard Deviation (SD) where applicable. GraphPad Prism 7 software (GraphPad Software) was used for statistical analysis. All the results are expressed as Mean ± SD.

Figures

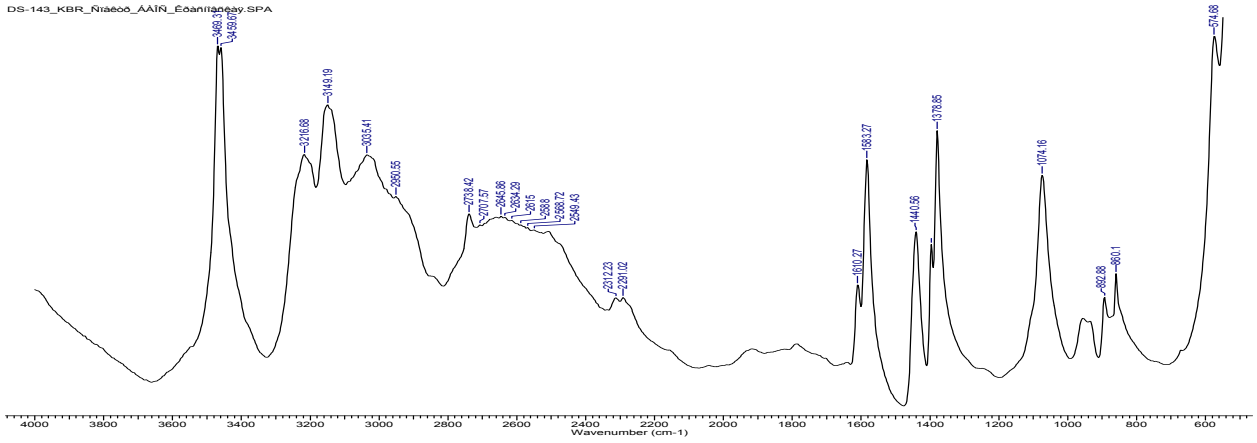


Figure S1. IR spectrum of oxoplatin 1.

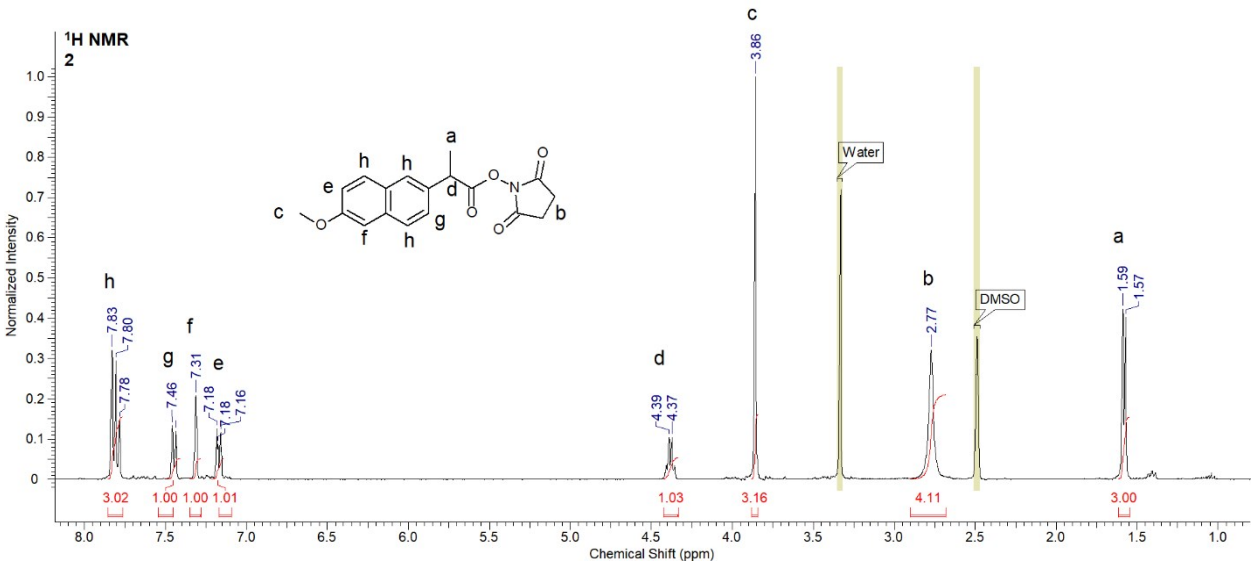


Figure S2. 1H NMR spectrum of 2.

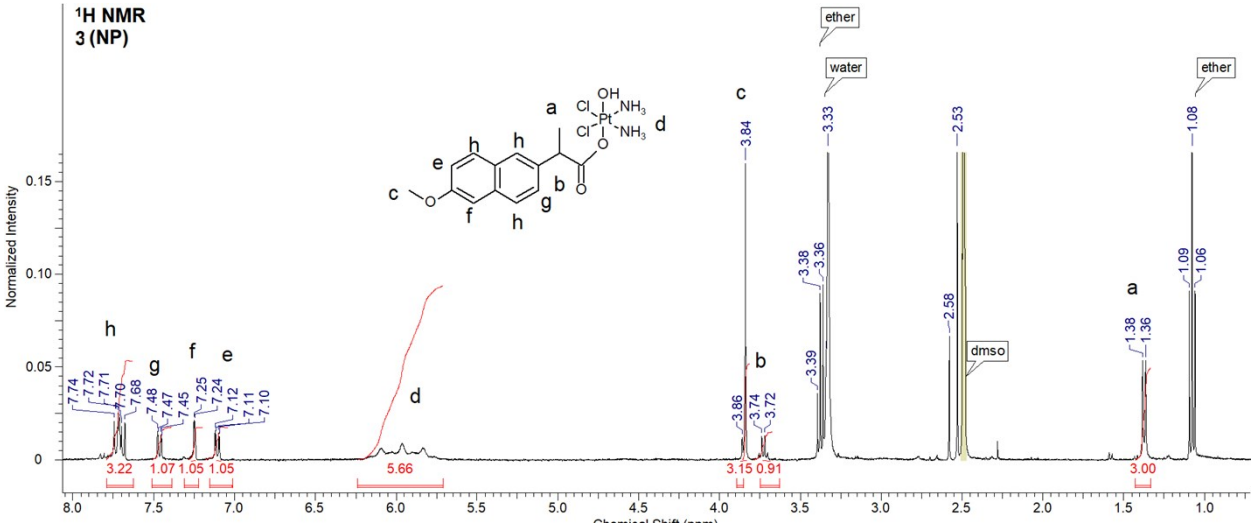


Figure S3. 1H NMR spectrum of NP.

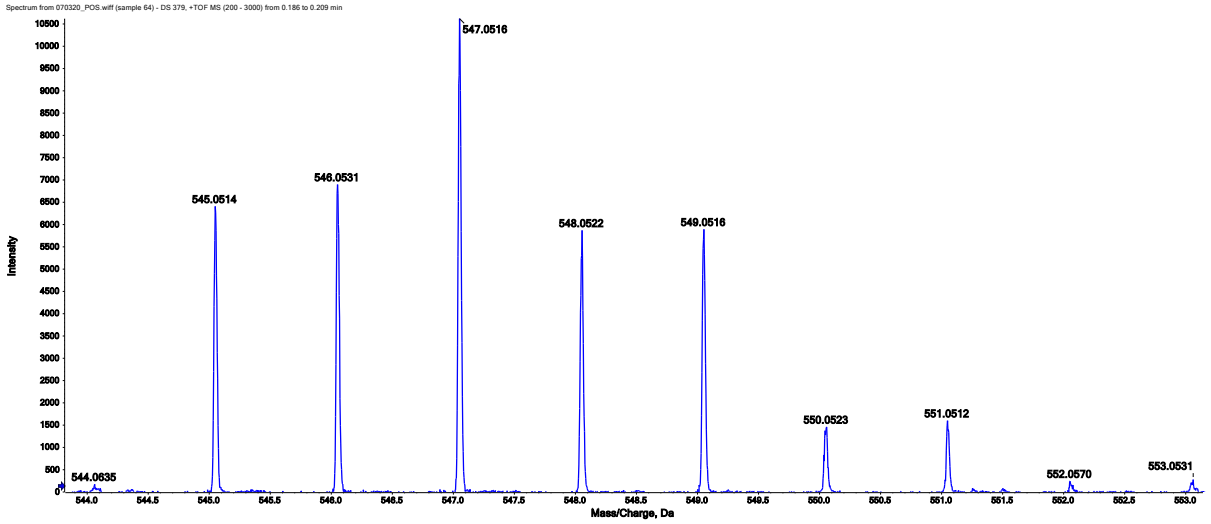


Figure S4. HRMS spectrum of NP.

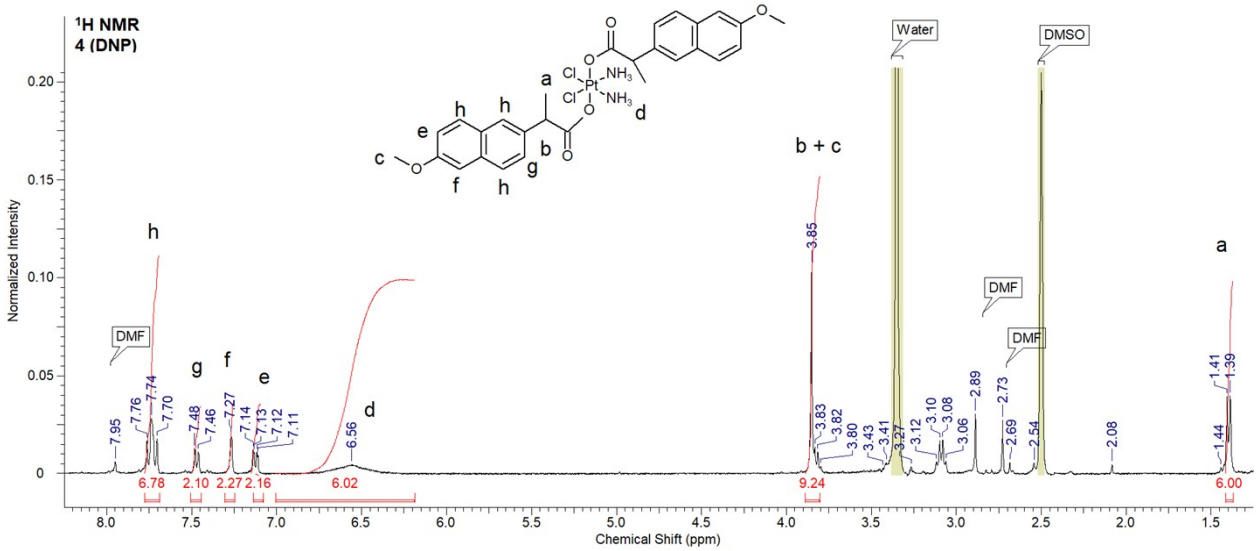


Figure S5. 1H NMR spectrum of DNP.

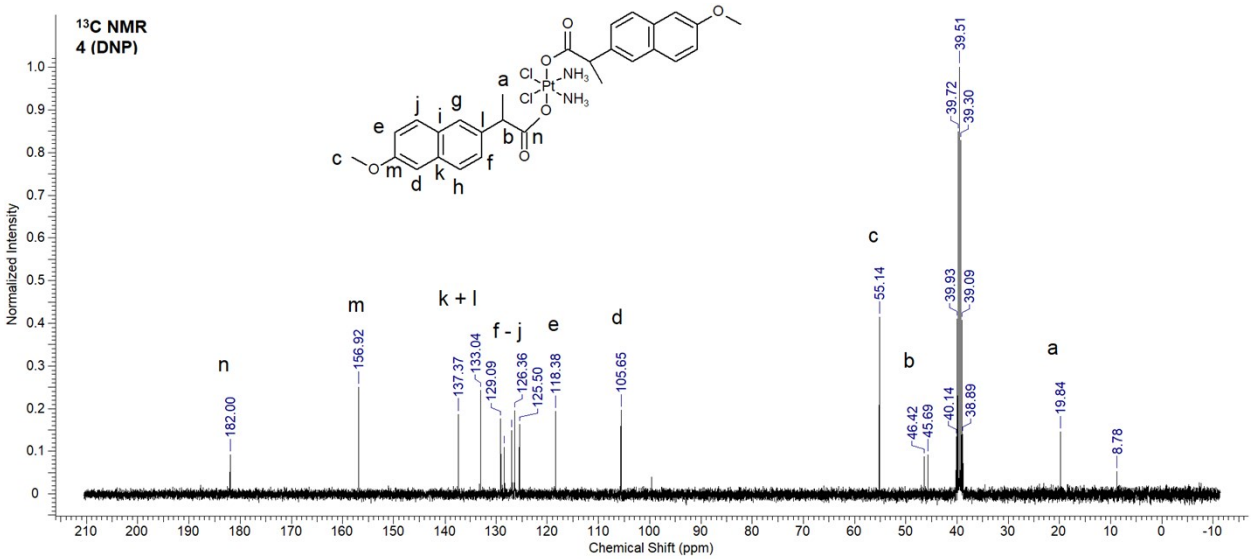


Figure S6. ^{13}C NMR spectrum of DNP

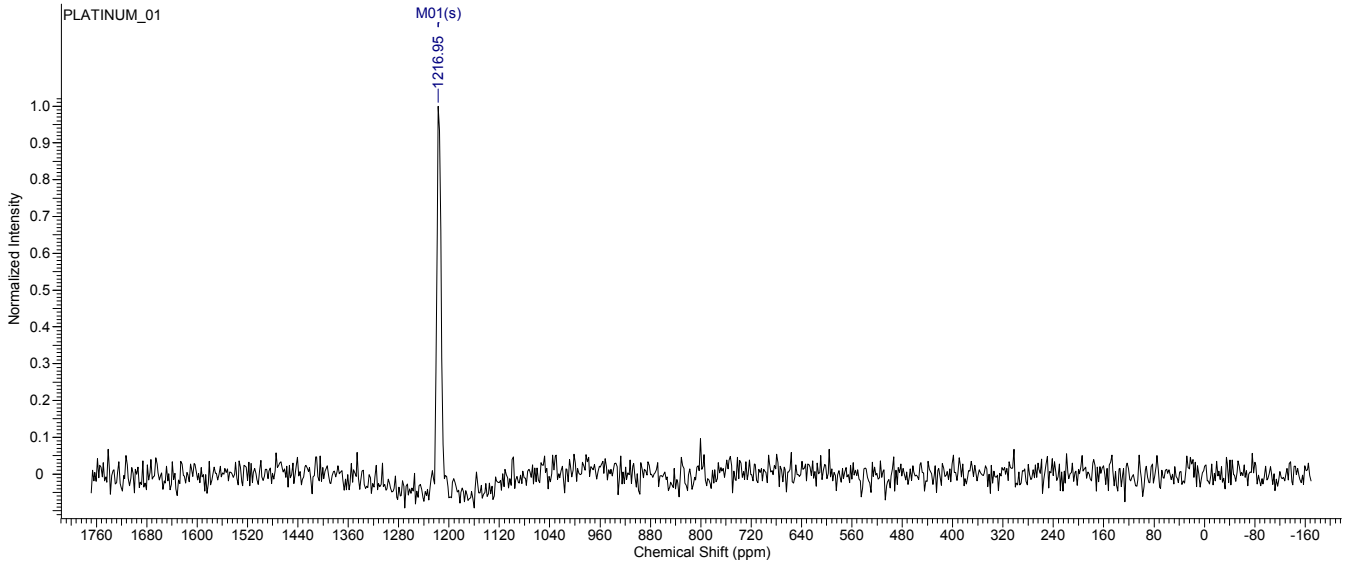


Figure S7. ^{195} Pt NMR spectrum of DNP

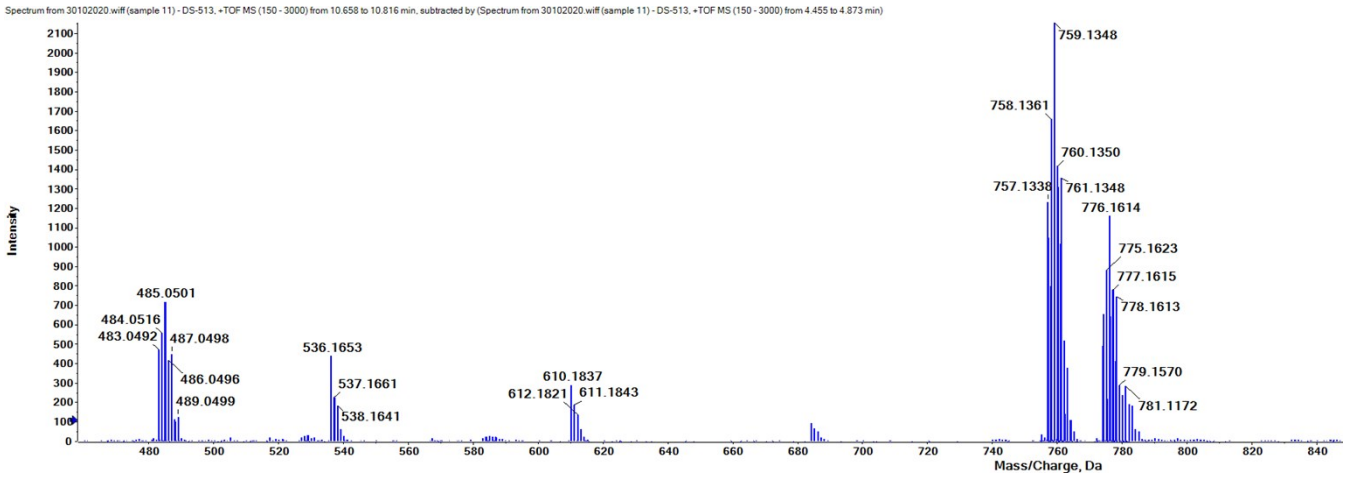
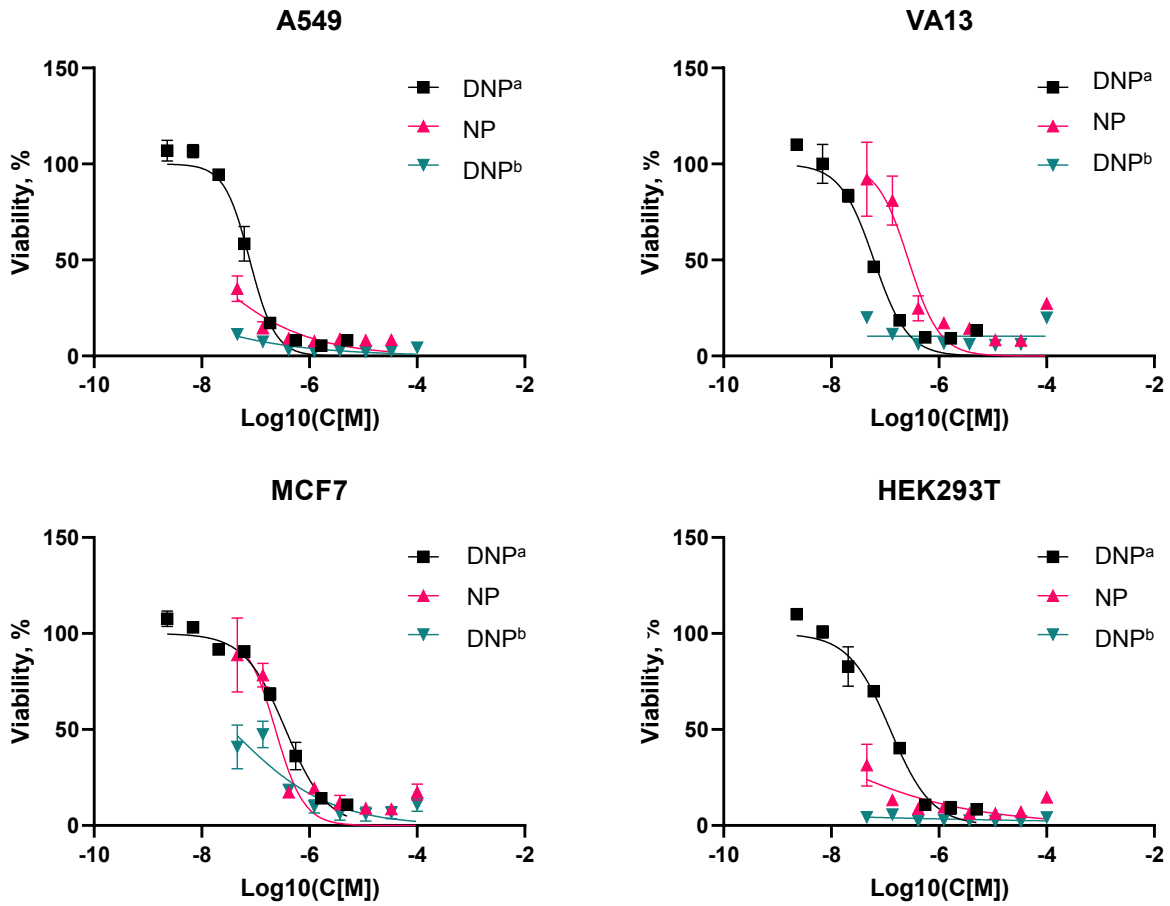
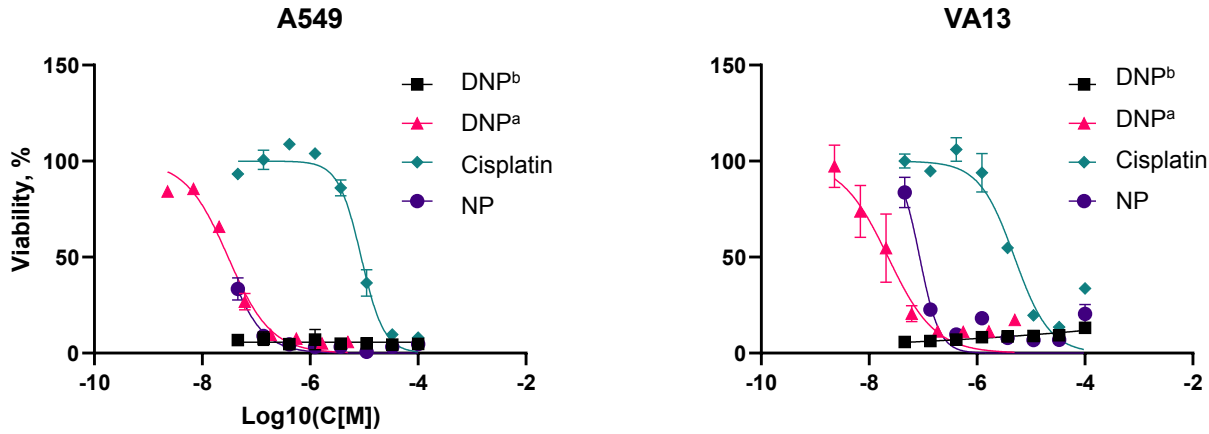


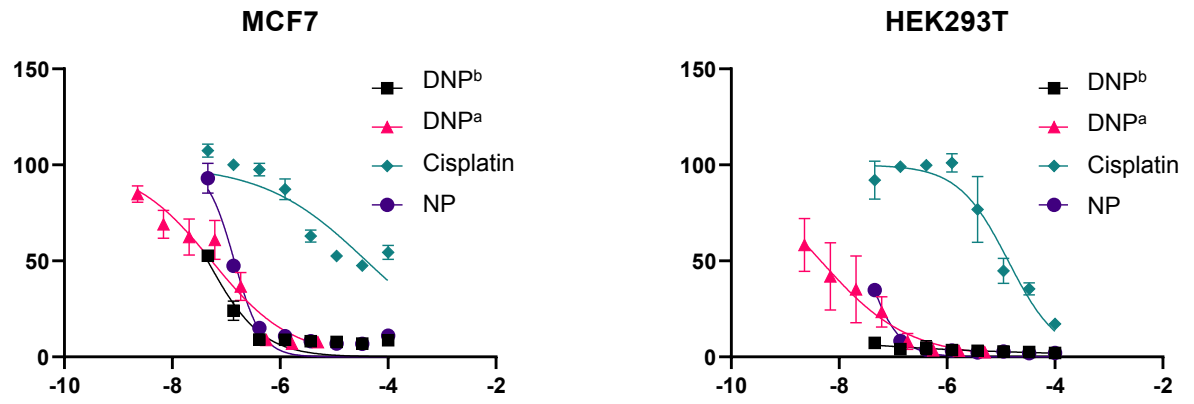
Figure S8. HRMS spectrum of DNP.



[a]initial concentration of DNP solution 1 mM
[b]initial concentration of DMP solution 20 mM

Figure S9. Cytotoxicity profile of NP and DNP against A549, Va13, MCF-7 and HEK293T cell lines.





[a]initial concentration of DNP solution 1 mM
[b]initial concentration of DMP solution 20 mM

Figure S10. Cytotoxicity profile of Cisplatin, NP and DNP against A549, Va13, MCF-7 and HEK293T cell lines.

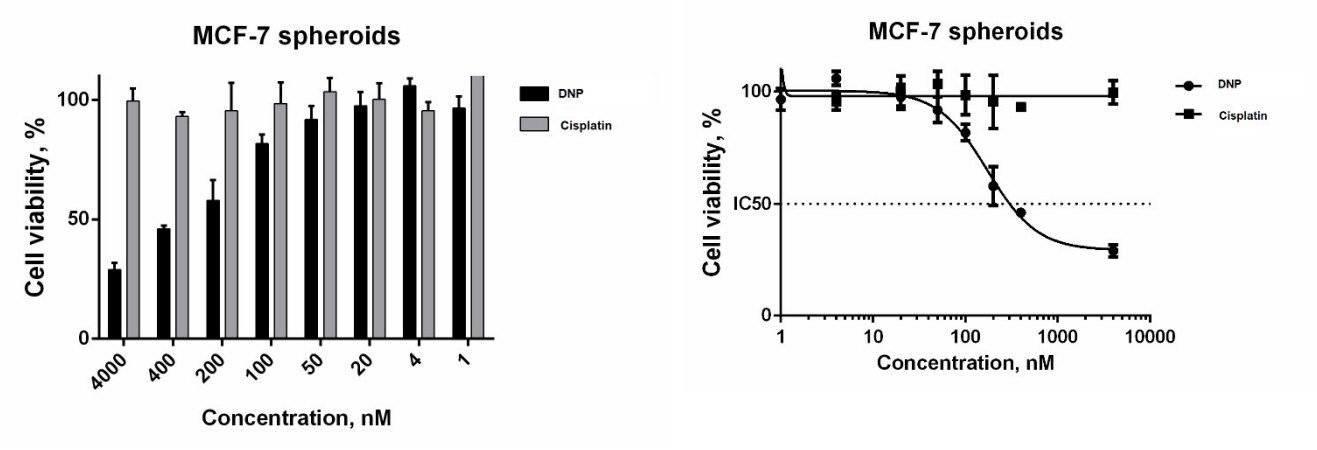


Figure S11. Cytotoxic activity of DNP and cisplatin on MCF-7 spheroids

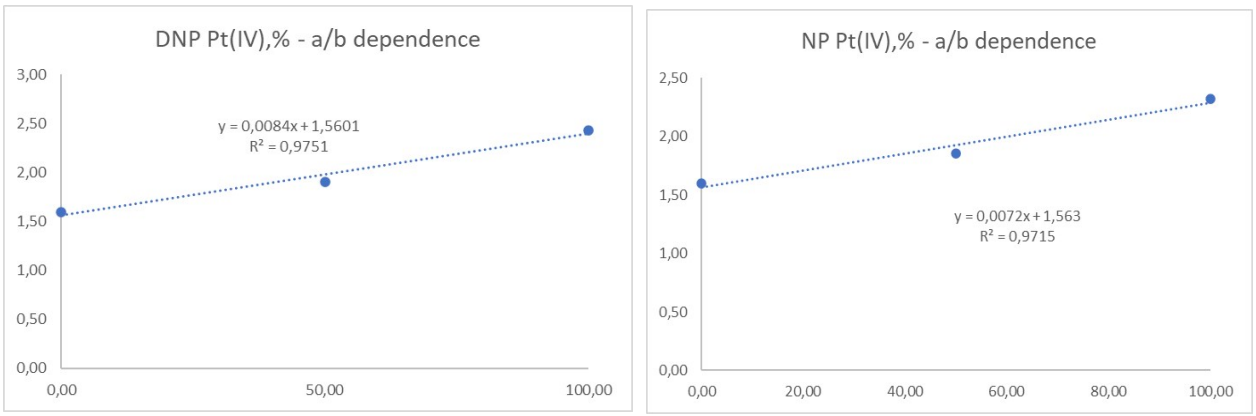


Figure S12. Linear dependence of *a/b* ratio for solid Pt^{IV} prodrugs, Cisplatin Pt^{II}, and equimolar mixture Pt^{II}+Pt^{IV} 50:50 for DNP (left) and NP (right).

Table S1. MTT data after 72 hours of incubation.

	Va13	**HEK293t**	**A549**	**MCF-7**
DNP	0,06±0.01	0,03±0.01	0,08±0,01	0,18±0.03
NP	0.28±0.05	0.115±0.01	0.28±0.01	0.19±0.02

Table S2. Cell cycle arrest by 0.2 μM of **DNP** (MCF-7 cells, 48 h of incubation).

	G1	S	G2
DNP	15,61	34,85	49,56
Control	28,69	42,07	29,25

Table S3. Intracellular accumulation and distribution of cisplatin and DNP in MCF-7 cells. Cells were incubated with 10 μM of compounds for 3.5 hours.

Compounds	**Cytoplasm**	**Mitochondria**	**Nucleus**
Cisplatin	0.022±0.015	0.018±0.014	0.008±0.008
DNP	0.831±0.272	0.854±0.03	0.063±0.039

References

[1] J. Friedrich, C. Seidel, R. Ebner, L.A. Kunz-Schughart, *Nature protocols*, **2009**, *4*, 309.

[2] Ryabaya, A. Prokofieva R. Akasov, D. Khochenkov, M. Emelyanova, S. Burov S,et al. *Biomed Pharmacother*. **2019,** *109*, 2548–2560.