Supporting Information

A Hydrolytically Stable Oxo-Rhenium(V) Antitumor Agent for Synergistic Combination Therapy with Cisplatin: From Synthesis and Mechanistic Studies to Toxicity Assessment in Zebrafish

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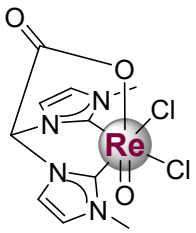
Materials.

All starting materials, reagents and solvents were of reagent grade quality or better, obtained from commercial suppliers, and used without further purification. Solvents were used as received or dried over molecular sieves. All preparations were carried out inside a fume hood using standard Schlenk techniques. The tripodal NHC ligand precursor 2,2-bis(3-methyl-1H-imidazol-3-ium-1-yl)acetate bromide (**L1**) was synthesized following a previously reported protocol.[1]

Instrumentation and methods.

^{1}H NMR and ^{13}C NMR spectroscopic measurements were done using a Varian 600 MHz or a Bruker 800 MHz spectrometers at 20 ºC in the NMR facility, TIFR Mumbai. The measurements were performed using standard deuterated solvents, chemical shifts δ are reported in ppm (parts per million), and coupling constants *J* (whenever appropriate) are noted as absolute values in Hz. The residual solvent peaks were considered as an internal reference for both ^{1}H and ^{13}C NMR spectra for expression of chemical shifts. Abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). Mass spectra were recorded either on a Bruker ultrafleXtreme MALDI-TOF or Thermo Q Exactive orbitrap mass spectrometer equipped with an electrospray ionization source. Simulated mass spectra were obtained from SISweb (https://www.sisweb.com/mstools.html). Inductively coupled mass spectrometry (ICP-MS) measurements were carried out using an Agilent 7900 ICP-MS. Analytical HPLC was performed using a Shimadzu-Nexera X2 UPLC system fitted with a C18 reverse-phase column (Waters C18, 2.1 mm × 50 mm, 1.7 μm). UPLC method: a linear gradient of Millipore water with 0.1% TFA (A) and acetonitrile (B, SigmaAldrich HPLC-grade) was used. t = 0.01 min, 10% B; t = 1.00 min, 15% B; t = 2.50 min, 20% B; t = 7.50 min, 100% B; t = 9.00 min, 100% B; t = 10.00 min, 10% B. The flow rates were 0.45 mL min^{-1}, and UV absorption was measured at 220/254 nm. . Elemental microanalyses were performed on a Thermo Fisher FLASH2000 CHNS/O analyzer.

Synthesis and characterization data of complexes 1-4.



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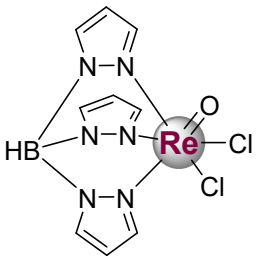
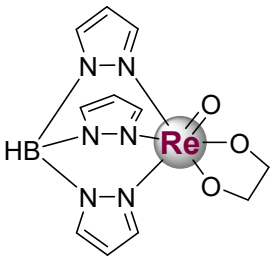
Complex 1. Complex **1** was synthesized following a literature procedure with slight modification.[2] Briefly, to a stirred solution of $[NBu_4][ReOCl_4]$ (50 mg, 0.08 mmol) in 2 mL MeOH was added ethylene glycol (17.9 mg, 0.29 mmol). The resultant brown coloured solution was stirred at room temperature for 15 min. Subsequently, suspensions of ligand precursor **L1** (36 mg, 0.13 mmol) in 1 mL MeCN and NaH (5 mg, 0.21 mmol) in 1 mL MeCN were added sequentially. The resulting solution was allowed to stir for 20 h at room temperature during which the colour of the solution changed to violet. Afterwards, excess of conc. HCl (5 mL, 37% in water) was added to the reaction mixture. The MeCN was removed under reduced pressure and the water phase was extracted with HFIP. Removal of HFIP under reduced pressure and addition of MeOH to the residue led to precipitation of a deep blue powder. It was washed with MeOH (2 x 1 mL) and Et_2O (2 x 1mL). Complex **1** was isolated as deep blue solid powder. Yield 4.5 mg, 11%. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 7.97 (d, J = 1.2 Hz, 2H), 7.85 (d, J = 1.2 Hz, 2H), 7.31 (s, 1H), 4.23 (s, 6H). ESI-MS (pos. detection mode): m/z 475.01 $[M\text{-}Cl+H_2O]^+$, calculated m/z for $[M\text{-}Cl+H_2O]^+$ 475.02.



2

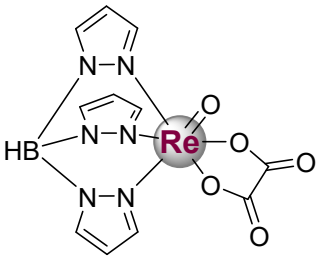
Complex 2. To a stirred solution of potassium tris(pyrazolyl)borohydride (0.86 gm, 3.41 mmol) in 30 mL EtOH was added conc. HCl (4 mL). The reaction mixture was stirred at room temperature for 15 min. Afterwards, $NaReO_4$ (0.20 gm, 0.73 mmol) was added and the reaction mixture was refluxed for 4 h. It acquired a blue colour during the course of the reaction. After completion of the reaction, it was allowed to cool to room temperature and flask was kept on

ice bath. The solid precipitated was filtered and subsequently washed with H_2O and EtOH to afford **2** as a blue powder. Yield 0.30 gm, 80%. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 8.42 (d, *J* = 2.4 Hz, 2H), 8.25 (d, *J* =1.8 Hz, 2H), 7.80 (d, *J* = 1.8 Hz, 1H), 7.31 (s, 1H), 6.76 (m, 2H), 6.11 (m, 1H). ^{13}C{^{1}H} NMR (201MHz, DMSO-d_6): δ (ppm) = 148.07, 146.92, 140.91, 135.91, 109.76, 106.68. ESI-MS (pos. detection mode): m/z 501.07 [(M-2Cl+2OH)+MeOH+H_2O+H]$^+$, calculated m/z for [(M-2Cl+2OH)+MeOH+H_2O+H]$^+$ 501.12. t_R (RP-UPLC) = 6.1 min. Elemental analysis calculated for **2**, $C_9H_{10}BCl_2N_6ORe$: C, 22.24; H, 2.07; N, 17.29. Found: C, 22.33; H, 1.95; N, 17.31.



3

Complex 3. To a solution of **2** (0.25 gm, 0.53 mmol) in MeCN (15 mL) were added Et_3N (292 µL, 2.1 mmol) and ethylene glycol (117 µL, 2.1 mmol) and the reaction mixture was refluxed with stirring for 24 h. After completion of the reaction, the acetonitrile was removed under reduced pressure and the residue was dissolved in dichloromethane (20 mL). The DCM layer was washed multiple times with water to remove the triethylamine hydrochloride and excess ethylene glycol. The dichloromethane layer was dried over Na_2SO_4 and concentrated *in vacuo.* The residue was subjected to silica gel column chromatography using EtOAc/hexanes (v/v, 3/2) as the eluting system to afford **3** as a purple-blue solid. Yield 0.15 gm, 70%. ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 8.24 (d, *J* =1.8 Hz, 2H), 7.92 (d, *J* = 1.8 Hz, 2H), 7.63 (d, *J* = 2.4 Hz, 1H), 7.55 (d, *J* = 1.8 Hz,1H), 6.51 (t, *J* = 2.4 Hz, 2H), 6.01 (t, *J* = 1.8 Hz,1H), 4.95-5.00 (m, 2H), 4.65-4.68 (m, 2H). ^{13}C{^{1}H} NMR (201 MHz, DMSO-d_6): δ (ppm) = 147.69, 141.55, 139.18, 134.79, 108.15, 105.35, 84.94. ESI-MS (pos. detection mode): m/z 499.06 [M+Na]$^+$, calculated m/z for [M+Na]$^+$ 499.07. t_R (RP-UPLC) = 4.2 min. Elemental analysis calculated for **3**·$(EtOAc)_{0.2}$, $C_{11.8}H_{15.6}BN_6O_{3.4}Re$: C, 28.75; H, 3.19; N, 17.05. Found: C, 28.57; H, 3.13; N, 16.82.



4

Complex 4. To a solution of **3** (0.1 gm, 0.21 mmol) in MeCN (15 mL) was added oxalic acid (0.023 gm, 0.25 mmol) and the reaction mixture was refluxed for 48 h. After completion of the reaction, the acetonitrile was removed under reduced pressure. The residue was subjected to silica gel column chromatography using EtOAc/hexanes (v/v, 7/3) as the eluent to afford **4** as a blue solid. Yield 0.05 gm, 50%. ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = 8.06 (d, *J* = 2.4 Hz, 2H), 8.01 (d, *J* = 1.8 Hz, 2H), 7.44 (d, *J* = 1.8 Hz, 1H), 7.19 (d, *J* = 1.8 Hz,1H), 6.57 (t, J=2.4 Hz, 2H), 6.01 (t, J=1.8 Hz,1H). ^{13}C{^{1}H} NMR (201 MHz, $CDCl_3$): δ (ppm) = 162.25, 148.69, 141.48, 140.68, 135.91, 109.60, 106.41. ESI-MS (pos. detection mode): m/z 501.08 [(M-oxalato+2OH)+MeOH+H_2O+H]$^+$, calculated m/z for [(M-oxalato+2OH)+MeOH+H_2O+H]$^+$ 501.12. t_R (RP-UPLC) = 4.4 min. Elemental analysis calculated for **4**·$(EtOAc)_{0.1}$, $C_{11.4}H_{10.8}BN_6O_{5.2}Re$: C, 26.74; H, 2.13; N, 16.41. Found: C, 26.66; H, 2.10; N, 16.02.

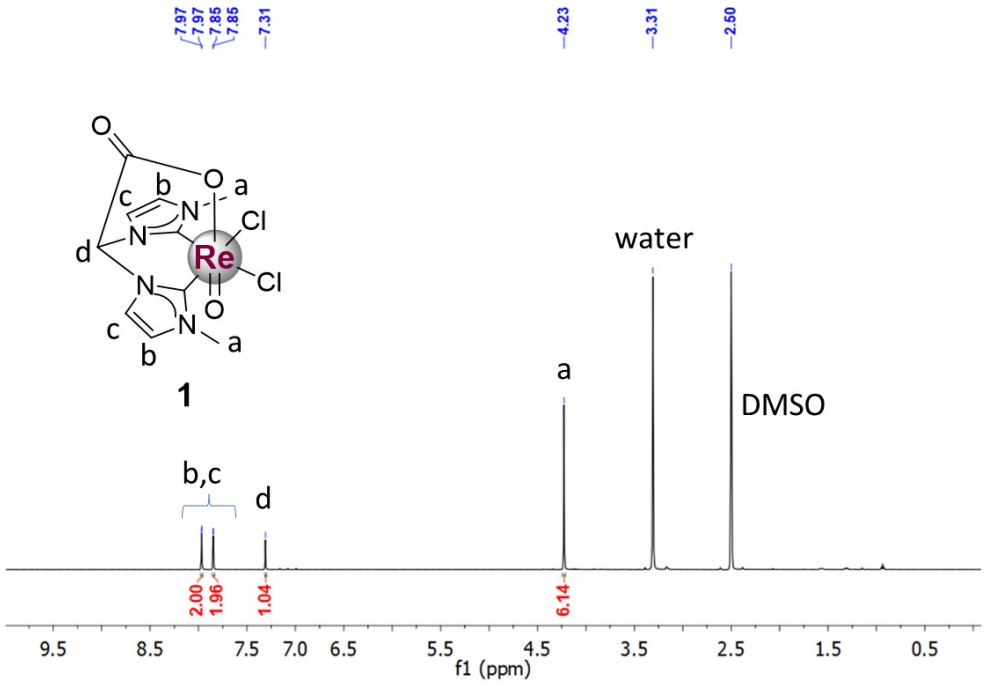


Figure S1. ^{1}H NMR of complex **1** in DMSO-d_6 (600 MHz)

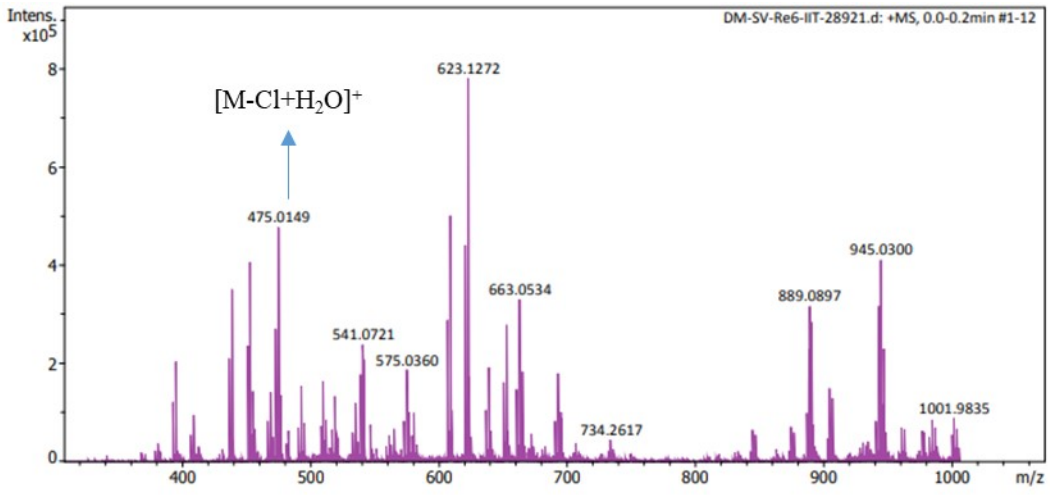


Figure S2. ESI-mass spectra of complex **1**.

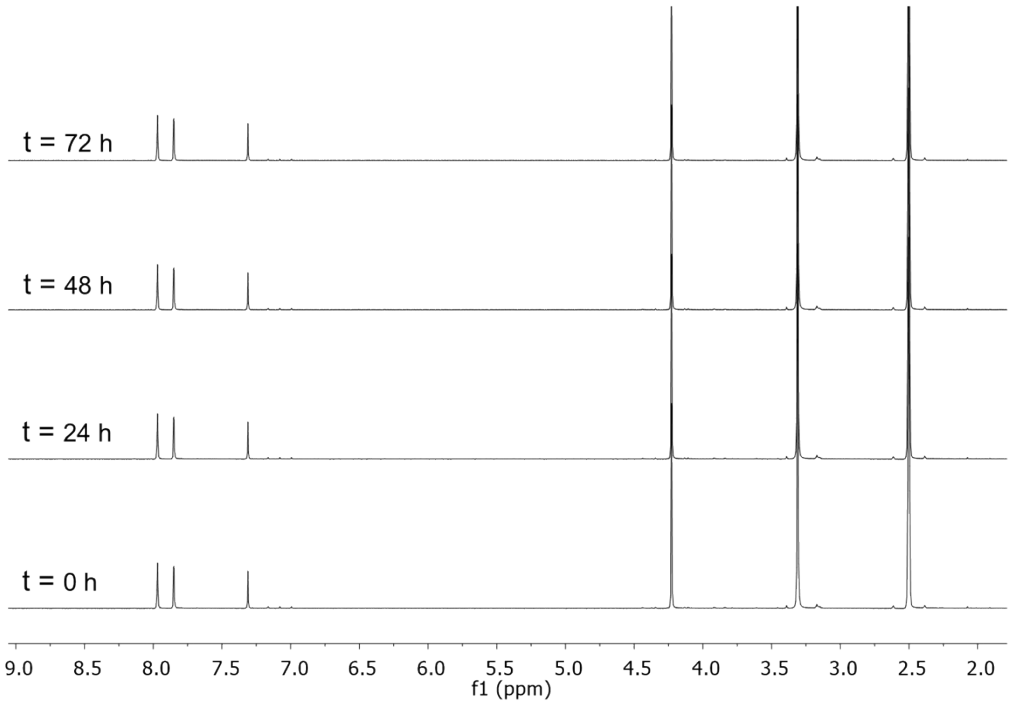


Figure S3. Time dependent ^{1}H NMR spectra of complex **1** in DMSO-d_6.

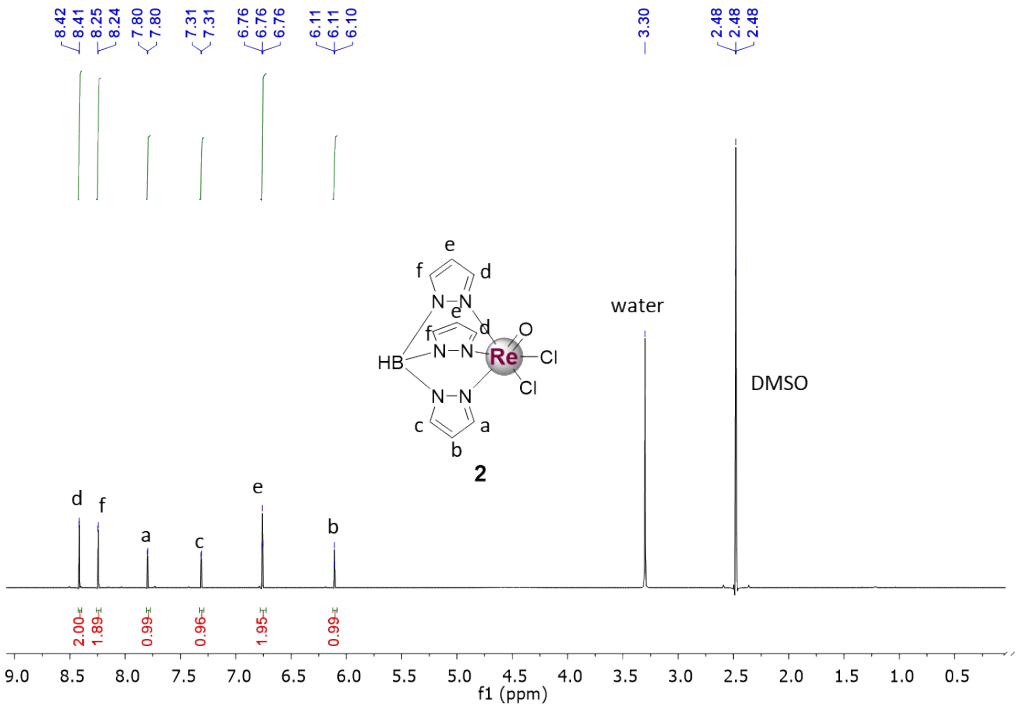


Figure S4. ^{1}H NMR spectrum of complex **2** (DMSO-d_6, 600 MHz).

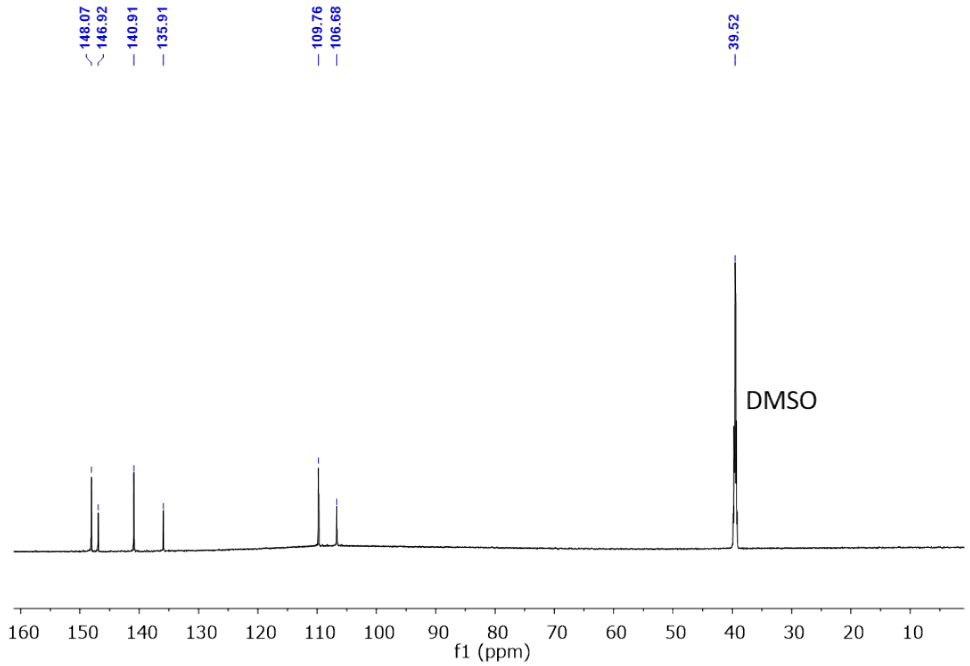


Figure S5. ^{13}C NMR spectrum of **2** (DMSO-d_6, 201 MHz).

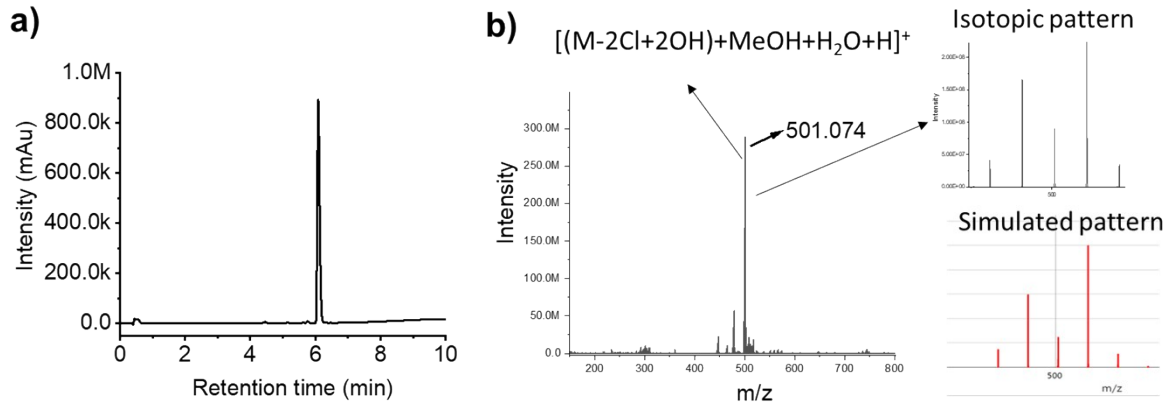


Figure S6. UPLC chromatogram at 254 nm **(a)** and ESI-mass spectrum in MeOH **(b)** of complex **2**.

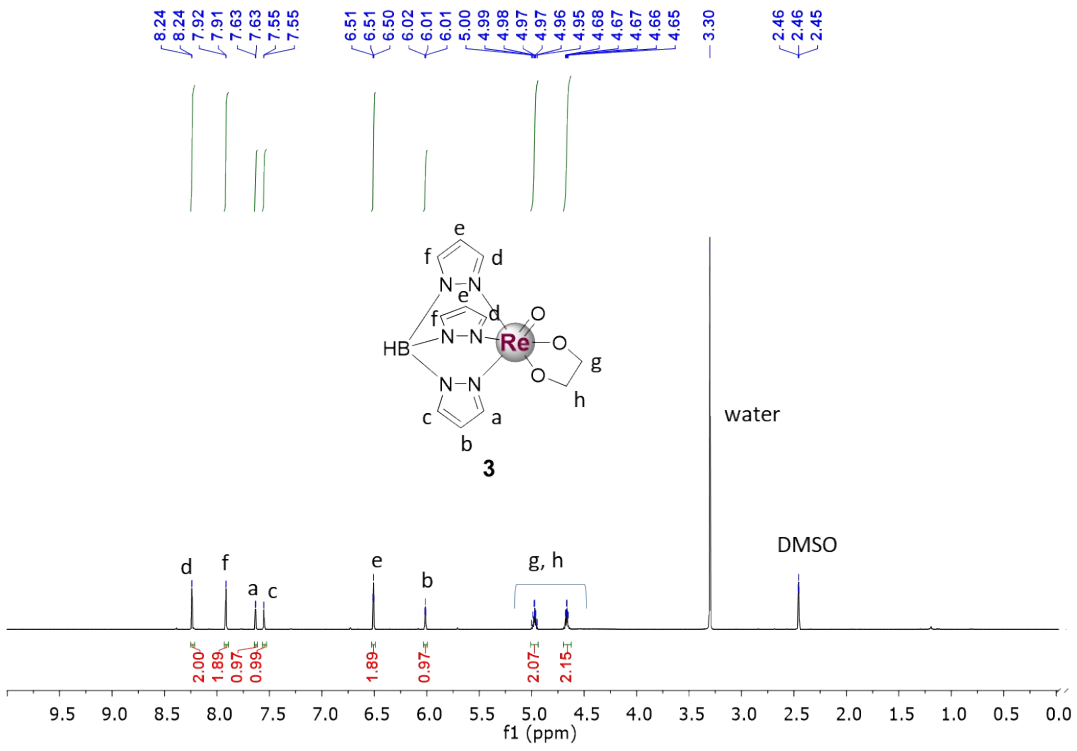


Figure S7. ^{1}H NMR spectrum of complex **3** (DMSO-d_6, 600 MHz).

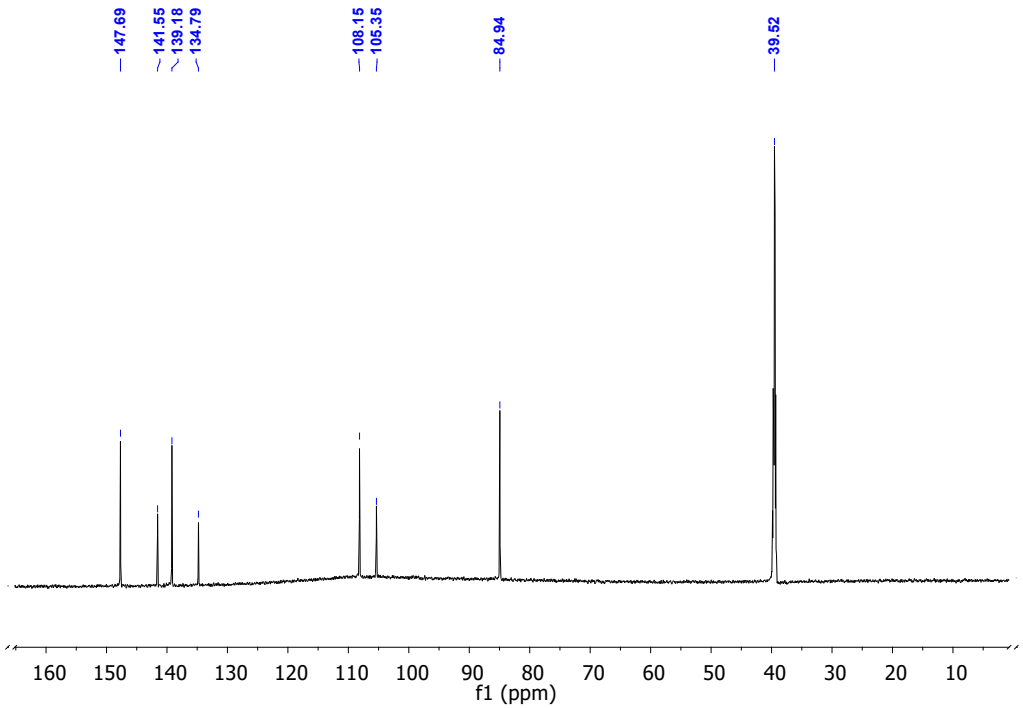


Figure S8. ^{13}C NMR spectrum of complex **3** (DMSO-d_6, 201 MHz).

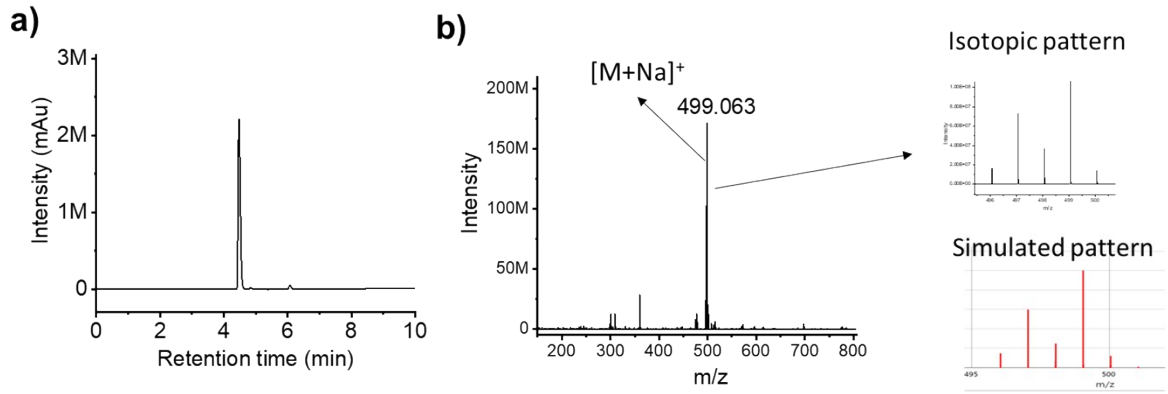


Figure S9. UPLC chromatogram at 254 nm **(a)** and ESI-mass spectrum in MeOH **(b)** of complex **3**.

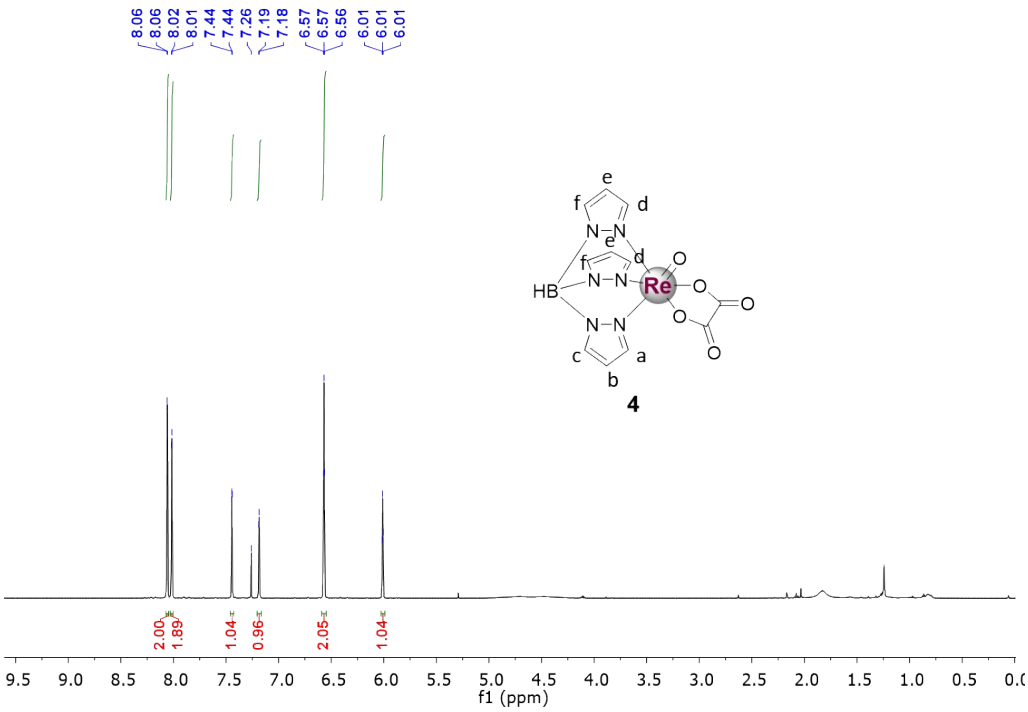


Figure S10. ^{1}H NMR spectrum of complex **4** ($CDCl_3$, 600 MHz).

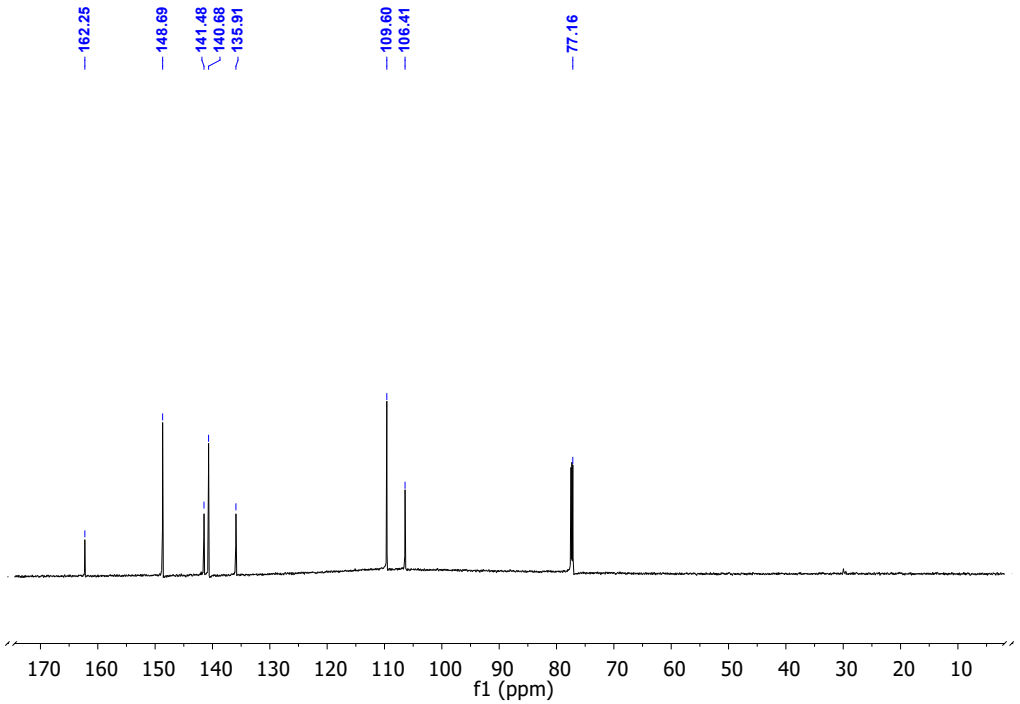


Figure S11. ^{13}C NMR spectrum of complex **4** ($CDCl_3$, 201 MHz).

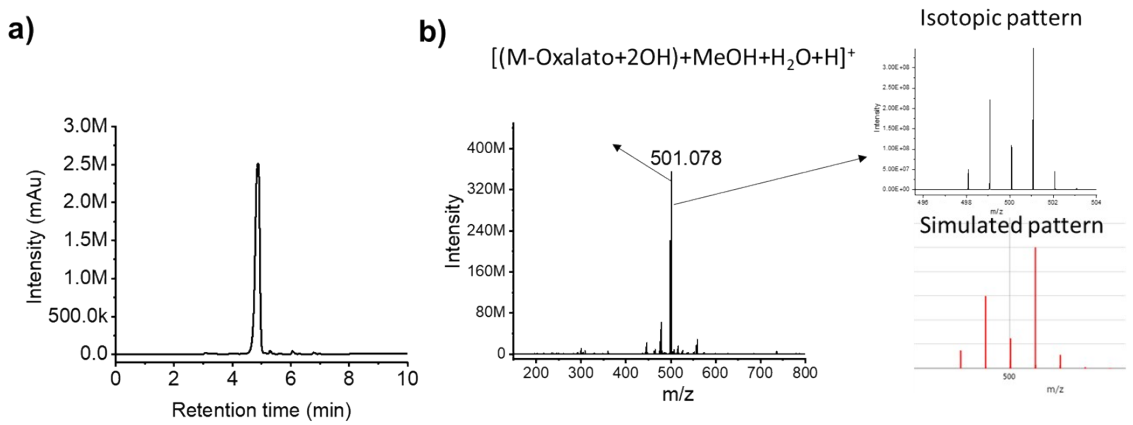


Figure S12. UPLC chromatogram at 254 nm **(a)** and ESI-mass spectrum in MeOH **(b)** of complex **4**.

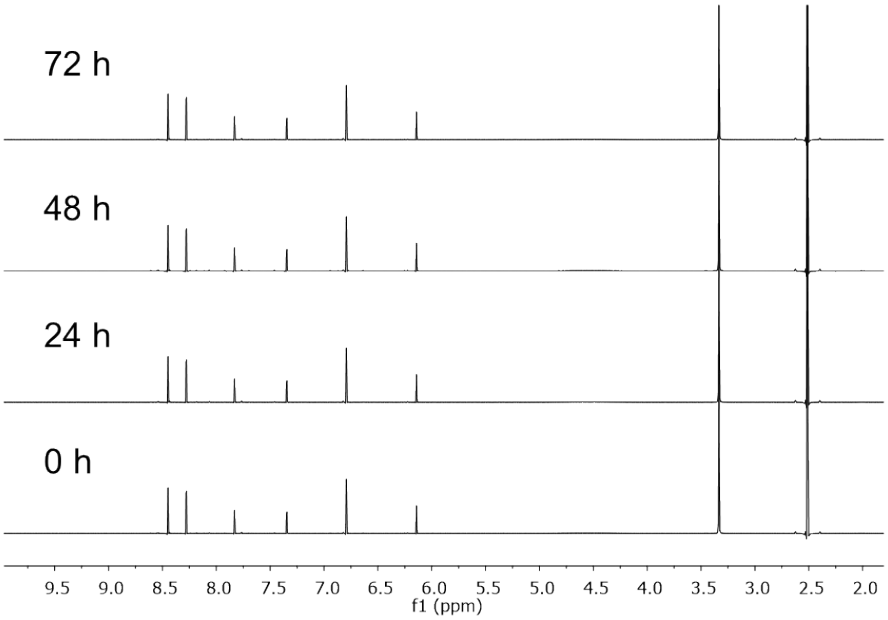


Figure S13. Time dependent ^{1}H NMR spectra of complex **2** in DMSO-d_6.

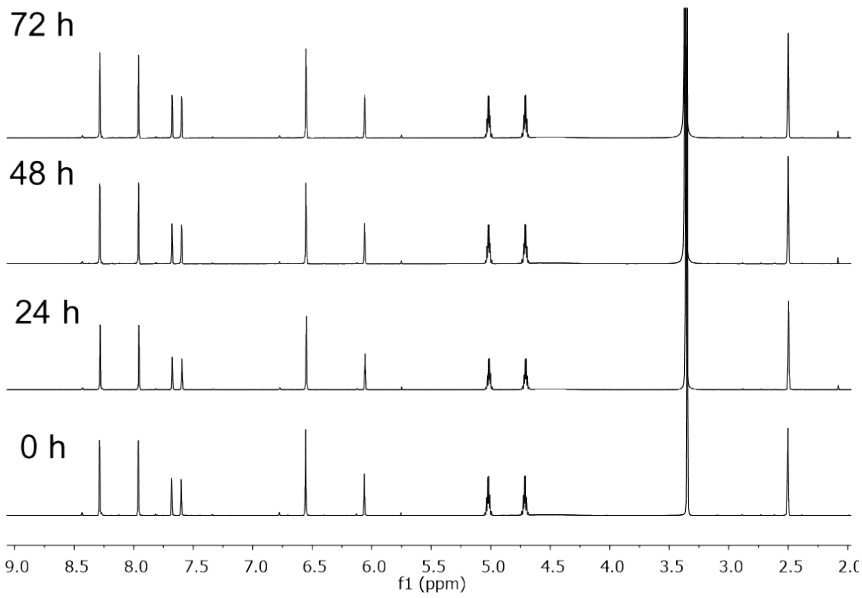


Figure S14. Time dependent ^{1}H NMR spectra of complex **3** in DMSO-d_6.

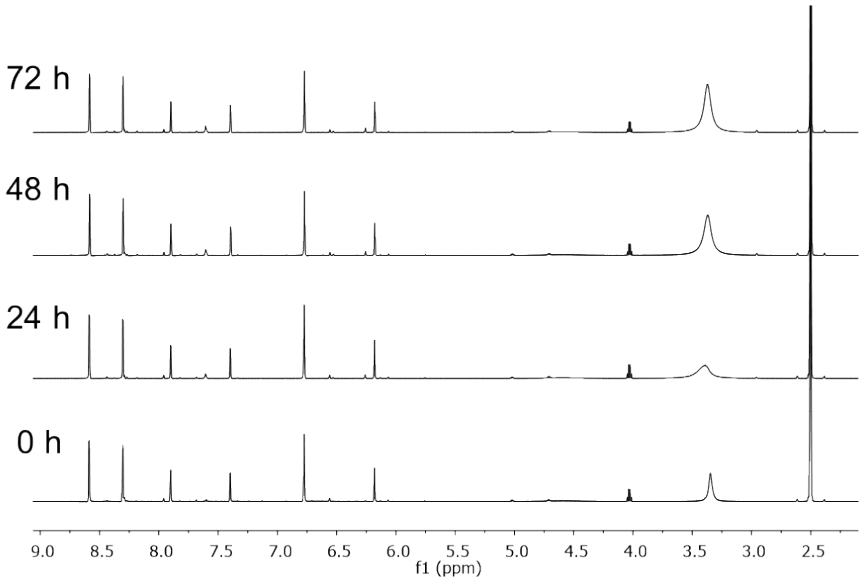


Figure S15. Time dependent ^{1}H NMR spectra of complex **4** in DMSO-d_6.

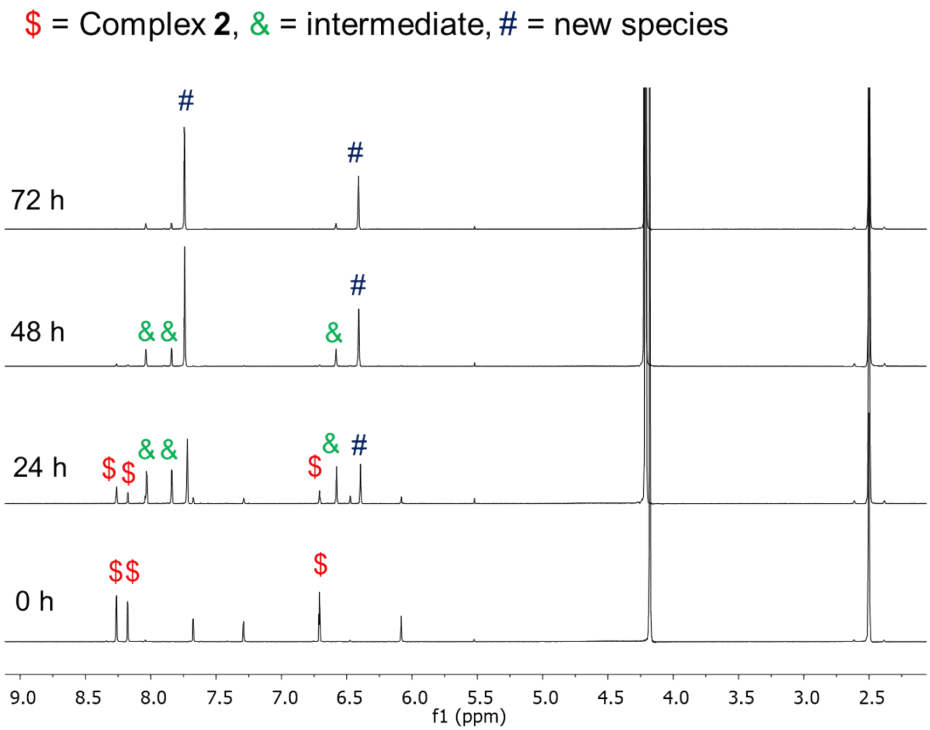


Figure S16. Time dependent ^{1}H NMR spectra of complex **2** in DMSO-d_6/D_2O (v/v 7/3).

Cell lines and cell culture maintenance.

HeLa cells are a generous gift from Stefano Ferrari's lab in the University of Zurich. A549, HT29, MDA-MB-231, DU145 and MRC5 cells were purchased from ATCC and ECAAC. DU145Cis (cisplatin resistant DU145) cell line was developed in our laboratory by exposing the parental DU145 cells to increasing concentration of cisplatin for more than an year.[3] All cell lines were expanded, stored in our laboratory tissue bank. Cells were checked for mycoplasma contamination before initiating the cultures. HeLa, A549 and MDA-MB-231 cells were grown using DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. DU145Cis and MRC5 cells were grown in EMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HT29 cells were grown in McCoy's 5A supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured in an incubator using 5% CO_2 with at 37°C.

Antitumor activity (MTT assay).

In vitro antitumor potency of the complexes was determined using colorimetric MTT assay. In a 96 well plate, 100 µL cell suspension per well containing 2500 cancerous cells or 7000 non-cancerous MRC5 cells were seeded and incubated for 20-24 h. The following day, fresh stock

solution of complexes was prepared in DMSO (for **1-4**) or in water (for cisplatin and carboplatin), exact concentrations were determined by quantifying metals using ICP-MS and immediately serially diluted using appropriate complete culture medium. The solutions were then added 100 µL per well (total volume per well 200 µL, final DMSO concentration was ≤ 0.25 % in treated as well as untreated control wells). The plate was then incubated for desired periods, 48h or 72 h. Subsequently, the media was replaced with 200 µL fresh media containing 0.4 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated further for 3h. The media was then aspirated and 180 µL/well of DMSO was added to lyse the cells and dissolve the resulting purple colored formazan crystals. The absorption was recorded at 570 nm using a BioTek Cytation 5 multi-well plate reader. The cell viability was calculated by normalizing each absorption value with respect to the untreated control and plotted against the applied concentrations using a Gen5 software to obtain the dose-response curve and IC_{50} values. The reported IC_{50} values are the average from at least three independent experiments, each of which consisted of either three or six replicates per concentration level.

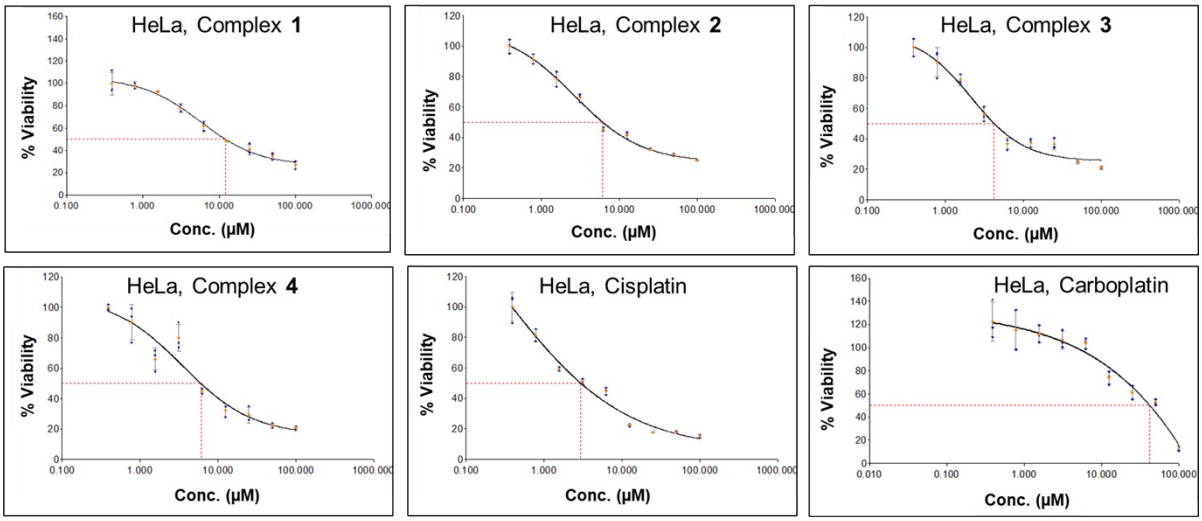


Figure S17. Dose-response plots of complexes **1-4**, cisplatin and carboplatin against HeLa cells (72 h MTT assay).

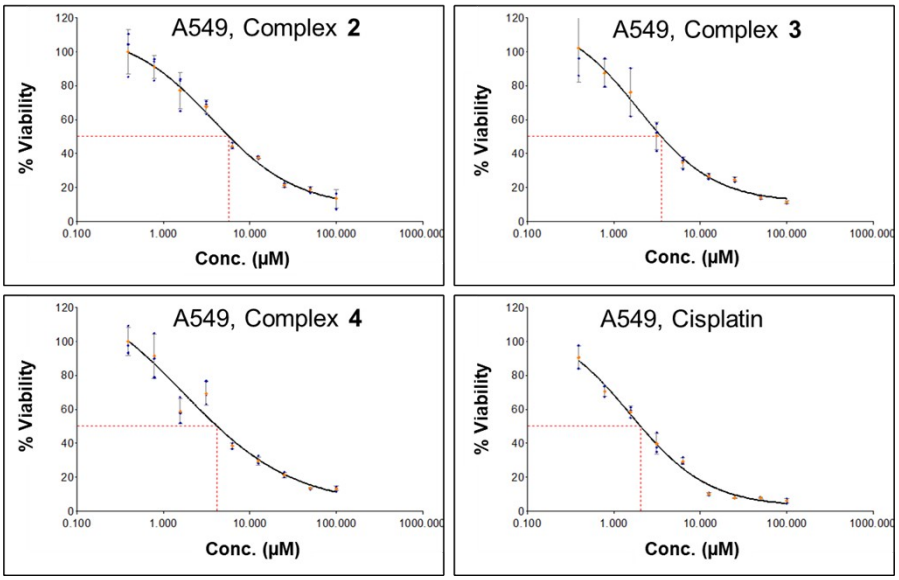


Figure S18. Dose-response plots of complexs **2**-**4** and cisplatin against A549 cells (72 h MTT assay).

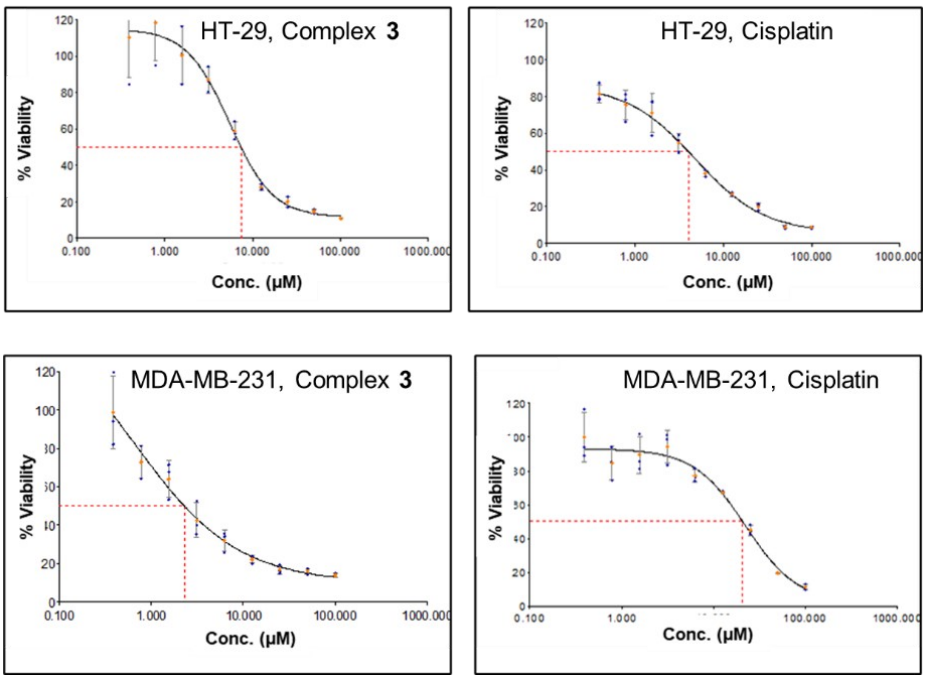


Figure S19. Dose-response plots of complex **2** and cisplatin against HT29 and MDA-MB-231 cells (72 h MTT assay).

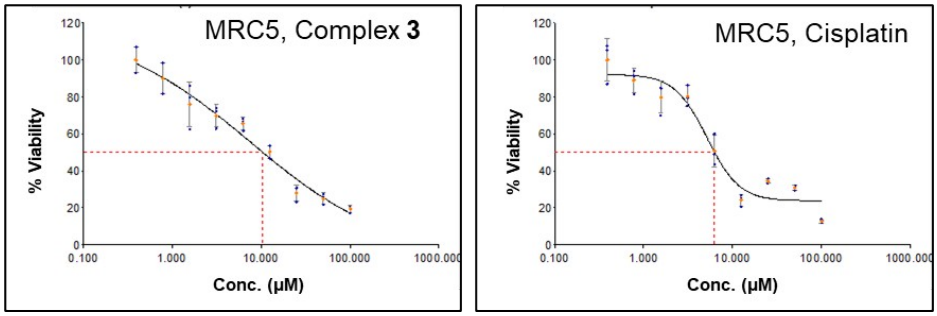


Figure S20. Dose-response plots of complex **2** and cisplatin against MRC5 (lung fibroblast) non-cancerous cells (72 h MTT assay).

Cell proliferation assay using trypan blue.

A549 cells were seeded in 24 well plates. Next day some of the wells were trypsinized, cells were collected, stained with trypan blue and live cells were counted using a hemocytometer to obtain the number of cells at 0 h. On the same day, some wells were left untreated and other wells were treated with different concentration of **3**. Number of viable cells in each well was counted at 24 h, 48 h, 72 h, 96 h and 120 h post incubation. The number of viable cells were plotted against incubation time using origin to obtain the proliferation plot in absence and presence of different concentration of **3**.

Measurement of lipophilicity (Log *P*).

Log*P* values of complexes **2-4** were determined from logarithmic ratio of distribution coefficient of complexes in an *n*-octanol and water biphasic mixture. Stock solutions of **2-4** were made in DMSO with a final concentration of 5mM. 6 µL aliquot from this stock solution was taken and the final volume was made to 500 µL. The octanol-water mixture with a final volume of 500 µL (1:1, v/v) was mixed for 1h at 37 °C using rotor rotating 70 rpm. The aqueous layer was carefully separated from the octanol layer. 75 µL of aqueous layer was digested with 300 µL nitric acid for 3 days at room temperature followed by 4 h at 60°C and the final volume was made to 5 ml using DI water. Similarly, 50 µL of octanol was taken and digested with 300 µL nitric acid for 3 days at room temperature followed by 4 h at 60°C. The final volume of the digested octanol layer was also made to 5 ml using DI water. The metal concentrations in both layers were measured using ICP-MS. The partition coefficients of metal complexes were calculated using the equation Log*P* = Log ([M] octanol /[M] water).

Cellular uptake measurement.

A549 cells (2.5 million) were seeded on 60 mm petri dishes and incubated for 24-36 h at 37 ºC. When the cells reached approximately 80-90% confluency, the medium was replaced with fresh 3.5 mL of fresh medium containing the test compounds (final concentration 6 μM). Cells were subsequently incubated for 6 h at 37 °C. The medium was then aspirated and cells were washed with PBS (3 x 3 mL). One of the petridish from each experimental condition was trypsinized (300 μL), cells were harvested and counted using a hemocytometer. The rest of the petri dishes were digested by addition 300 μL ICP-MS grade 70% HNO_3 (incubation, 2 days at room temperature) followed by 300 μL H_2O_2 (incubation, 1 day at room temperature). Samples were then collected in 15 mL tubes, diluted using double distilled water and metal contents were analyzed by ICP-MS to obtain the whole-cell uptake which was normalized with protein content.

Interaction of complex 3 with biomolecules.

Compound **3** (500 μM) and the relevant biomolecules (*N*-acetyl cysteine NAC, as small molecule model for biothiols and guanosine 5′-monophosphate GMP, as small molecule model for DNA) (5 mM) were mixed in an acetonitrile/water mixture (1:1, v/v) and incubated at 37 °C in a rotor at 30 rpm. 5 μL from that mixture was injected into the UPLC at regular time intervals up to 72 h. Carbamazepine (400 μM) was employed as the internal standard. The mixture obtained after 72 h of incubation was used for ESI-MS analysis (positive mode), and the molecular ion peaks of the metal-biomolecule adducts were assigned accordingly.

Intracellular organelle distribution of complex 3.

A549 cells (5 x 10^6) were seeded in a 100 mm culture plate and allowed to grow overnight. Then the cells were treated with 10 μM of compound **3** for 48 h. Media aspirated, plates were washed with 3x 5 mL PBS and cells were collected by trypsinization and pelleted. Cells were then resuspended in 1 mL PBS, counted and re-pelleted. The nuclear and cytoplasmic fractions were isolated using NEPER kit (ThermoFisher) following manufacturer protocol. Protein content and metal content in each fraction were measured using BCA assay and ICP-MS, respectively.

For quantifying accumulation of compounds in mitochondria, a similar protocol was followed after isolating and purifying the mitochondria using a mitochondria isolation kit (ThermoFisher) as per manufacturer's instructions. The metal content was normalized with the

protein content from the respective samples. Each of the experiments was conducted three times with three or more replicates.

DNA Metalation in A549 cells.

A549 cells (5 x 10^6) were seeded in a 100 mm culture plate and allowed to grow overnight. The cells were treated with 20 μM compound **3** or 5 μM cisplatin (as positive control) for 48 h. Then media was aspirated, cells were washed with PBS (3x5 mL) and then DNAzol (1 mL, genomic DNA isolation reagent, ThermoFisher) was added to the petridish and the lysate was transferred into a 1.5 mL tube. The DNA was precipitated with pure ethanol (0.5 mL), washed with 75% ethanol (0.75 mL × 3), and re-dissolved in 500 μL of 8 mM NaOH. The DNA concentration was determined by SpectraMax QuickDrop Micro-Volume Spectrophotometer and the respective metal content was measured using ICPMS. The experiment was performed in triplicates.

JC-1 assay.

A549 cells (0.75 million) were seeded in 35 mm tissue culture plates (2 mL media) and allowed to grow for ~24 h. Then the media was replaced with fresh media containing different concentrations of **3** (10 μM, 20 μM or 40 μM) and incubated for 48 hr. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, Sigma) was used as a positive control (50 μM, 30 min incubation). Then the media was aspirated, the cells were washed with 1x PBS (2 x 2 mL), harvested by trypsinization, pelleted, washed with ice-cold PBS (0.5 mL). The cell pellet was resuspended in 0.5 mL of PBS and to the tube was added JC-1 dye (2 μM) and incubated for 25 min in the dark at 37 °C in 5% CO_2. Subsequently, the cells were acquired using BD FACS Aria Fusion SORP system. The data were analyzed using Flowjo Software V10.

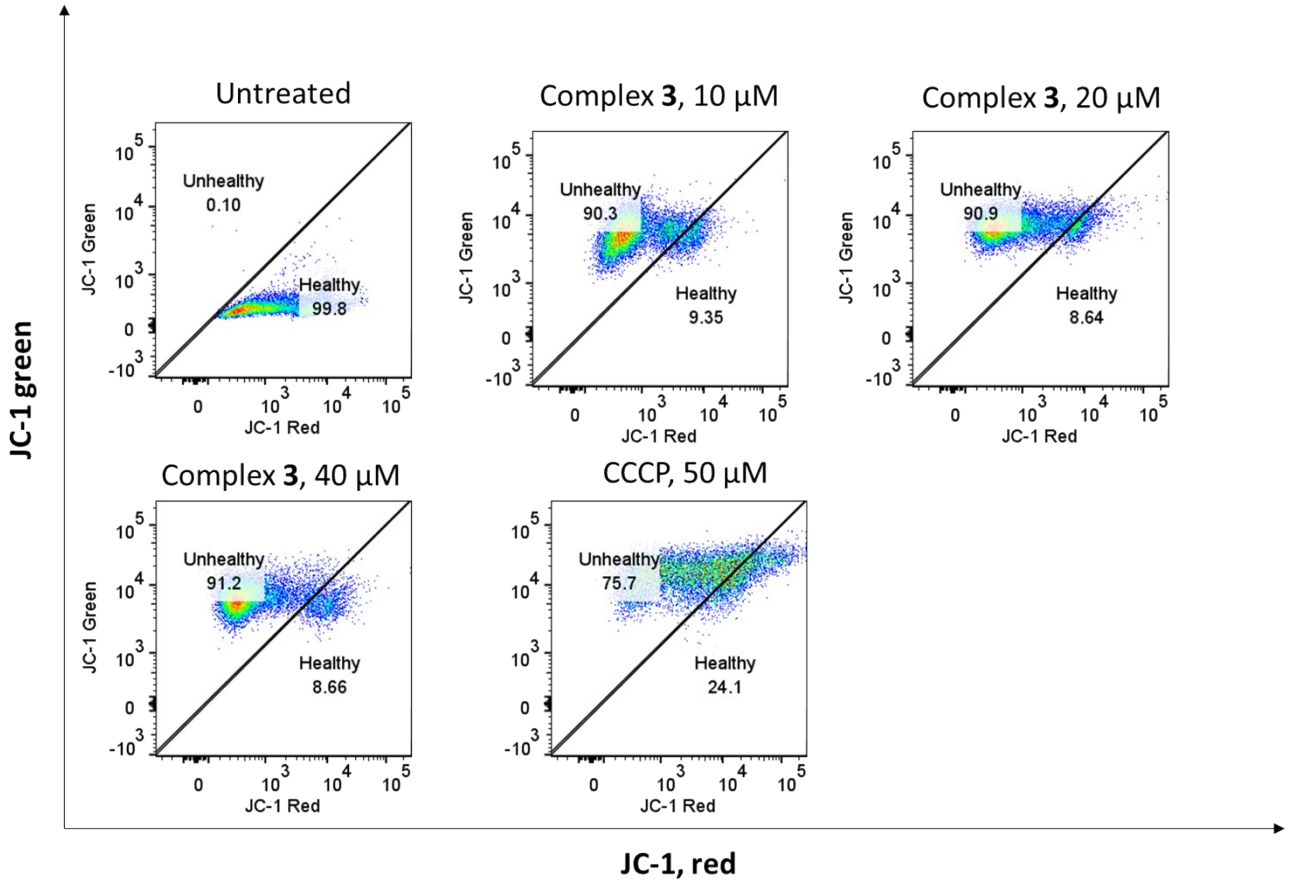


Figure S21. Representative JC-1 flow cytometry dot plot of A549 cells treated with 10-40 µM ($0.5xIC_{50}$/48h – $2xIC_{50}$/48h) of complex **3** for 48 h or CCCP (positive control, 50 µM, 15 min).

Intracellular superoxide and total ROS quantification.

A549 cells were seeded at a density of 0.75×10^6 cells in a 6 well plate and allowed to grow overnight. Cells were then treated with 10 µM to 40 µM compound **3** for 48 hrs. Luperox (500 µM, 30 min incubation) was included as positive control. The cells were harvested by trypsinization, washed with ice-cold PBS, centrifuged at 1200 rpm for 5 min at 4 °C and the supernatant was discarded. Cells were then incubated with DHE (final conc.10 µM in 100 µL PBS for 30 min at 37 °C in an incubator. Then 400 µL PBS was added to the cell suspension, centrifuged and supernatant was discarded. The cell pellet was resuspended in 300 µL PBS and acquired immediately using fluorescence-activated cell sorting (FACS) on a BD FACS Aria Fusion SORP system). Approximately 20,000 cells were acquired and the data were analyzed using FlowJo Software V10.

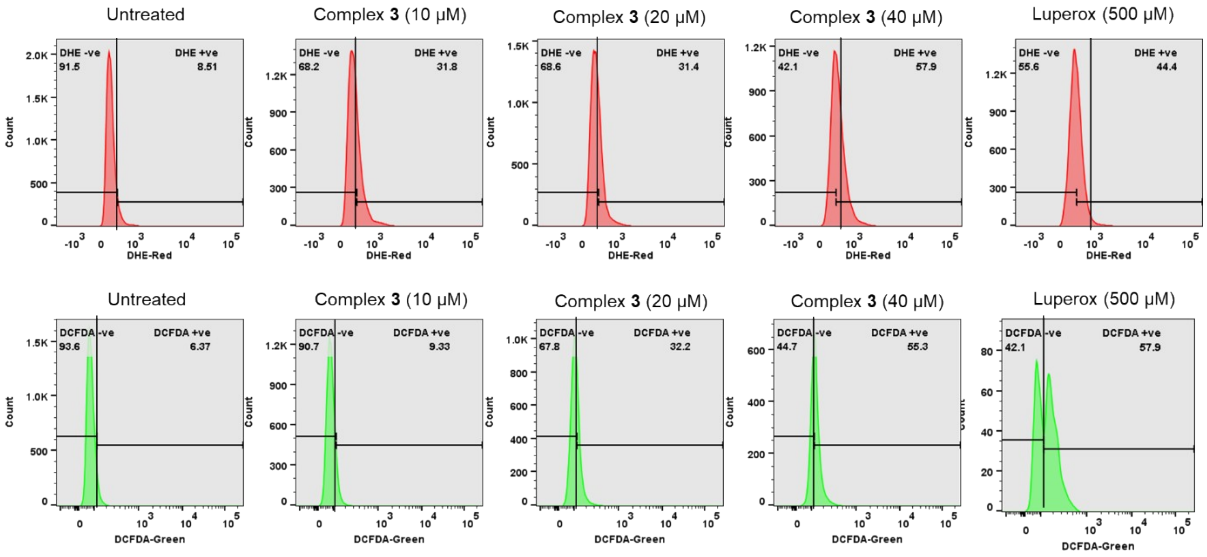


Figure S22. Representative flowcytometry histogram showing the red fluorescence from DHE stained (top panel) or H_2DCF-DA stained (bottom panel) A549 cells either kept untreated or treated with complex **3** with 10 µM - 40 µM (0.5x$IC_{50/48h}$ - 2x$IC_{50/48h}$) for 48 hrs exposure. Luperox (500 µM, 0.5 h) was used as positive control.

Immunoblotting analysis.

Approximately one million A549 cells were seeded in a 60 mm petri dish. When the confluency reached 70%, cells were treated with desired concentrations of testcompounds for 48hrs. Medium was then aspirated, cells were washed with ice-cold 1XPBS, scrapped into 150 µL of RIPA lysis buffer containing 1x protease inhibitors. The lysate was then centrifuged and protein concentration was calculated using BCA assay, then remaining supernatant was mixed with 4x Laemmli buffer (final concentration 1.25x) and incubated at 90ºC for 10 min. The cell lysates were then resolved by 10 % and 12 % sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; 120-150 V for 60 min) followed by electro-transfer to a polyvinylidene difluoride membrane (PVDF) (300 mA for 2.5 h). Membranes were blocked using 2 % (w/v) skim milk in TBSfor 30 min., the solution was decanted, and the membrane was incubated with the primary antibody in the ratio of 1:1000 primary antibodies in TBS overnight at 4 °C. On the following day, after washing with TBST (0.1 % Tween 20 in TBS) (3×2 mL), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) in TBS in the ratio of 1:1000. Immune complexes were detected with the ECL detection reagent (Thermofisher) in the Biorad Chemidoc system. List of primary antibodies used from Cell Signaling Technology: PARP Antibody (9542L), Cleaved

Caspase-3 (9664L), γ-H2AX (2577L), RIP1 Antibody (4926S), RIP3 (95702S), Phospho-eIF2α (3398S), IRE1α (3294S), Ubiquitin (43124S), LC3B Antibody (2775S), GAPDH (2118L) and Secondary antibody: HRP-linked Antibody-7074S (Cell Signaling Technology).

Analysis of Annexin-V-APC/PI double-stained A549 cells by flow cytometry.

A549 cells were seeded (0.75×10^6 cells) in 35 mm Petri plates and kept them in incubator overnight prior to compound incubation (at 37 °C with 5% CO_2). Cells were then treated with the compound **3** (10 μM, 20 μM or 40 μM, 48 h), or cisplatin (10 μM, 24 h) or H_2O_2 (10 mM, 15 min) and incubated at 37 °C with 5% CO_2. The cells were harvested by trypsinization, washed with ice-cold 1x PBS, centrifuged at 1200 rpm for 5 min at 4°C. Cell pellet was resuspended in 500 μL of Annexin-V binding buffer (1x buffer) and washed by centrifugation at 1200 rpm for 5 min at 4 °C. The cells were then resuspended in 350 μL of 1x Annexin-V binding buffer. 5 μL of Annexin-V-APC, 2 μL propidium iodide (1 mg/mL) were added and cells were incubated at 37 °C for 15 min. Then 150 μL of 1x Annexin-V binding buffer was added and centrifuged at 1200 rpm for 5 min at 4 °C and supereminent was discarded. The pellet was suspended in 300 μL 1x PBS and cells were acquired immediately with BD FACS Aria Fusion SORP system. The data were analyzed using Flowjo Software V10.

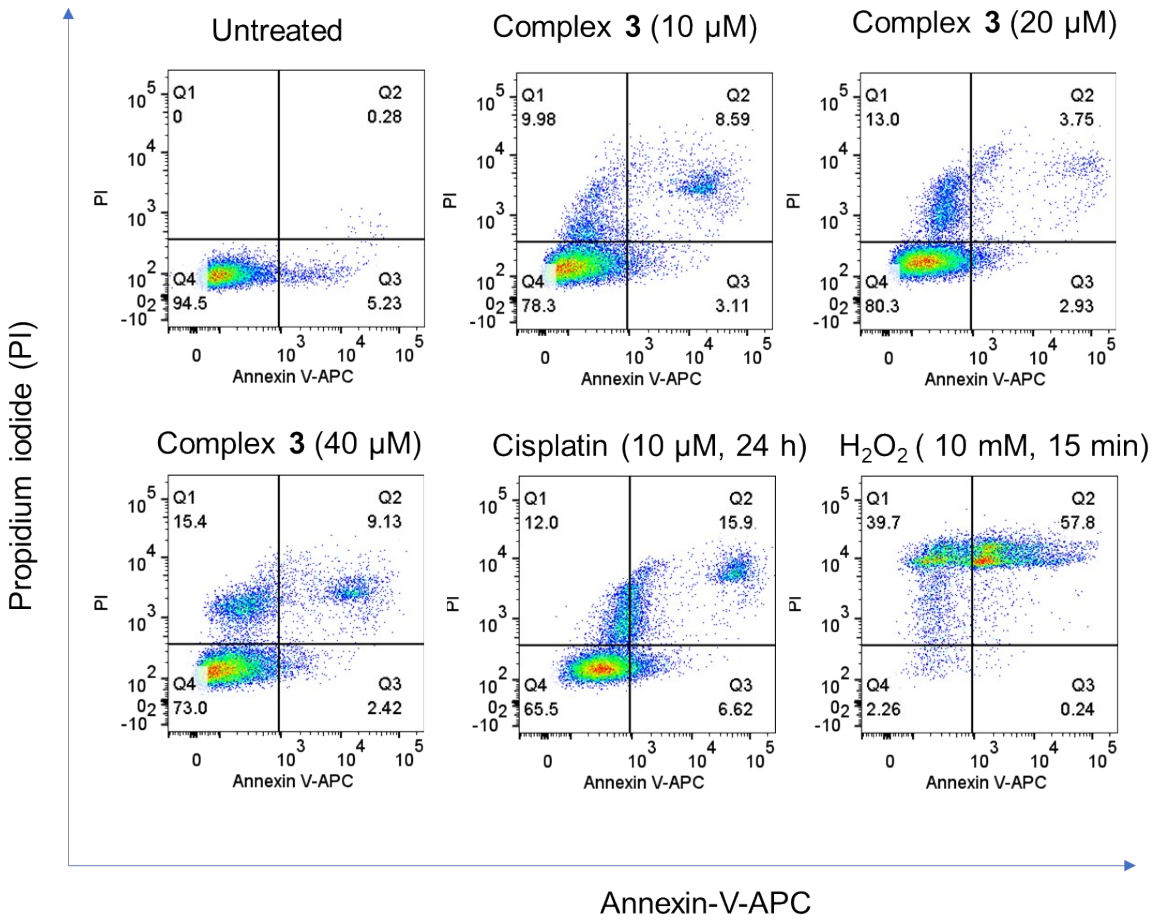


Figure S23. Representative flow cytometry dot plots of A549 cells treated with 10-40 µM ($0.5xIC_{50/48h}$ – $2xIC_{50/48h}$) of complex **3** for 48 h, 10 µM cisplatin for 24 h and H_2O_2 (10 mM, 15 min).

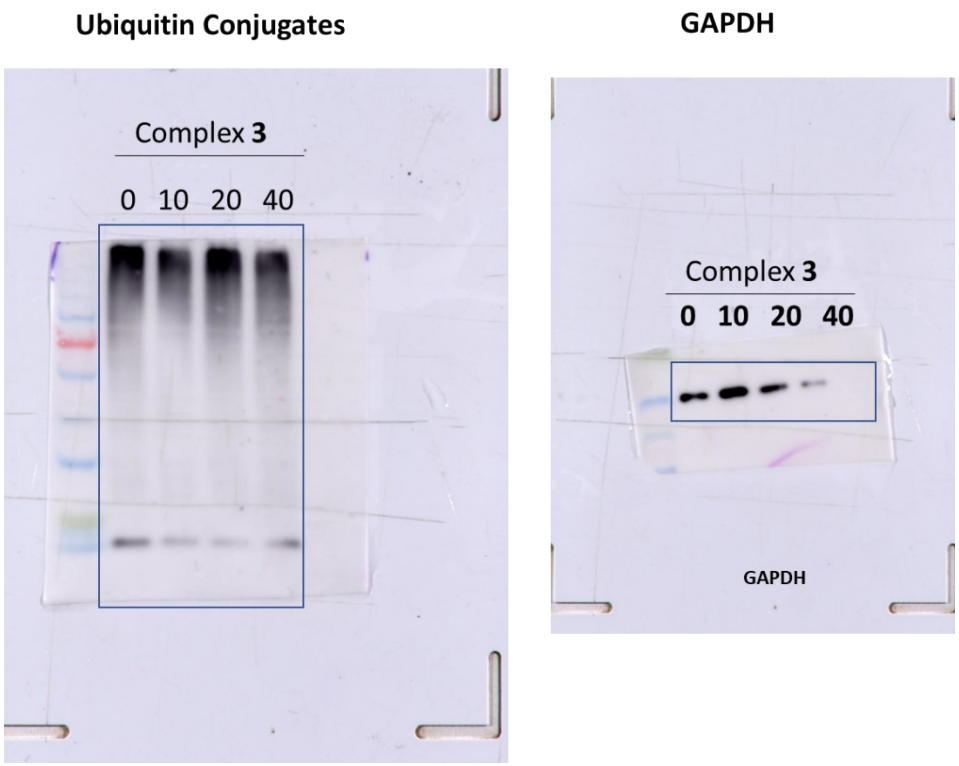


Figure S24. Uncropped full blots for Figure 10a.

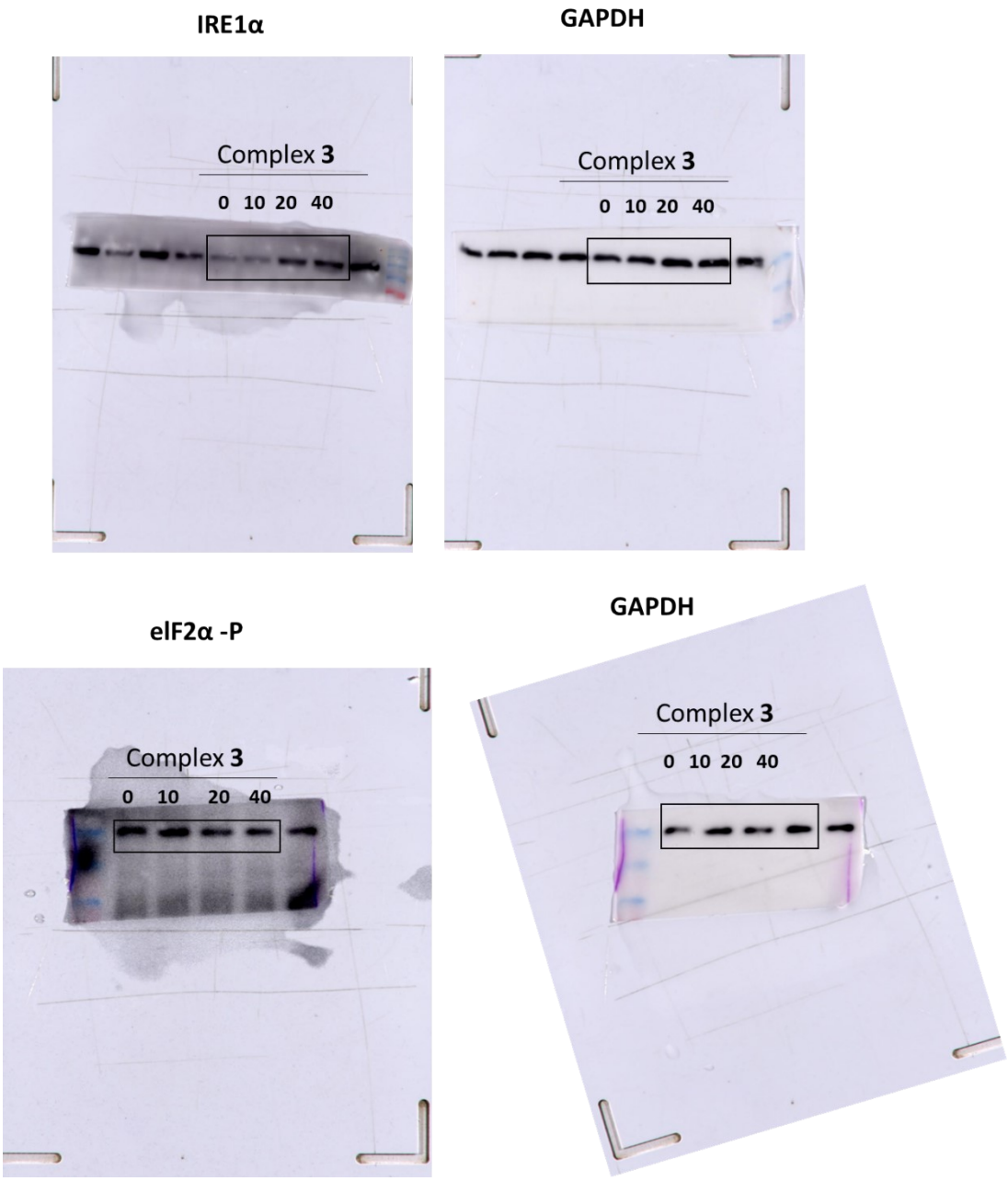


Figure S25. Uncropped full blots for Figure 10b.

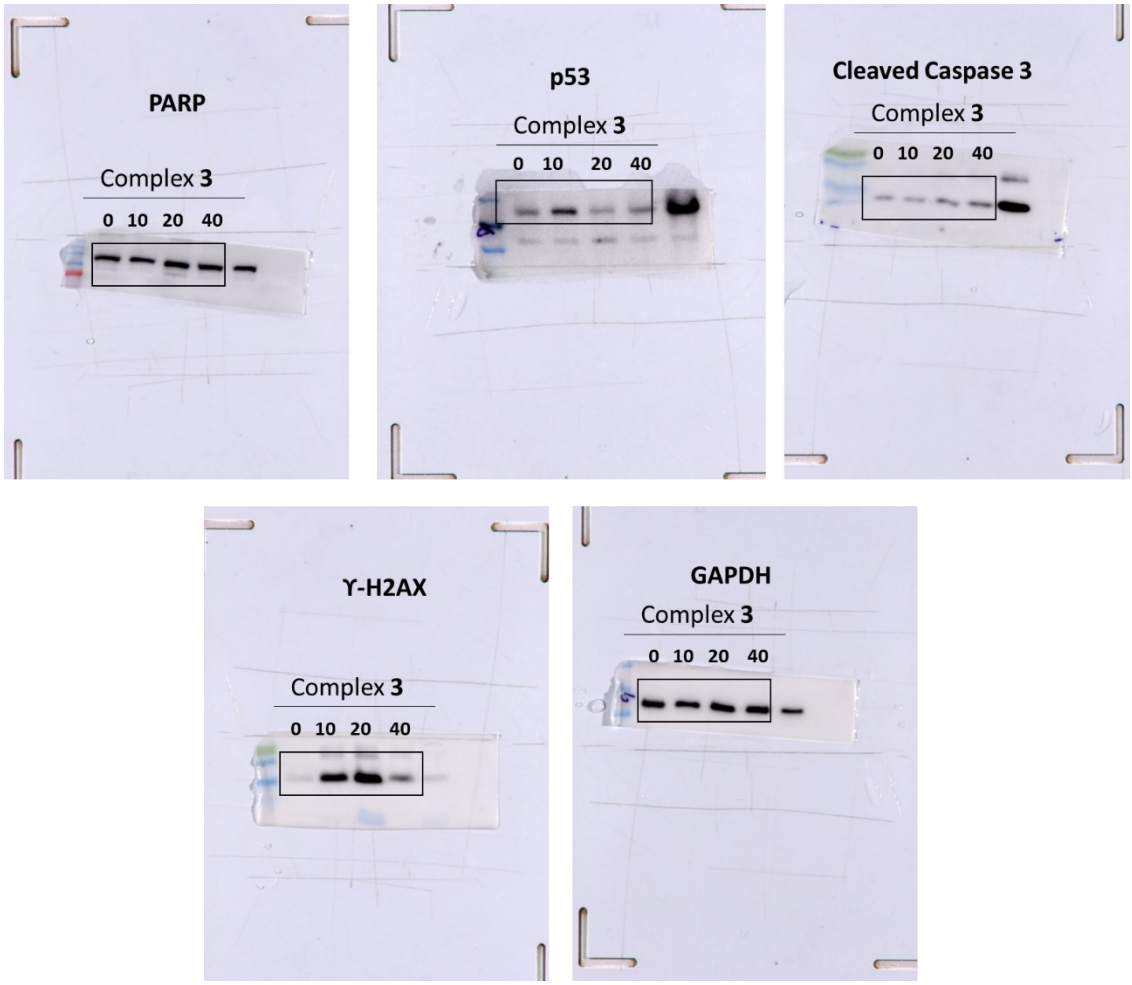


Figure S26. Uncropped full blots for Figures 7c and 11b.

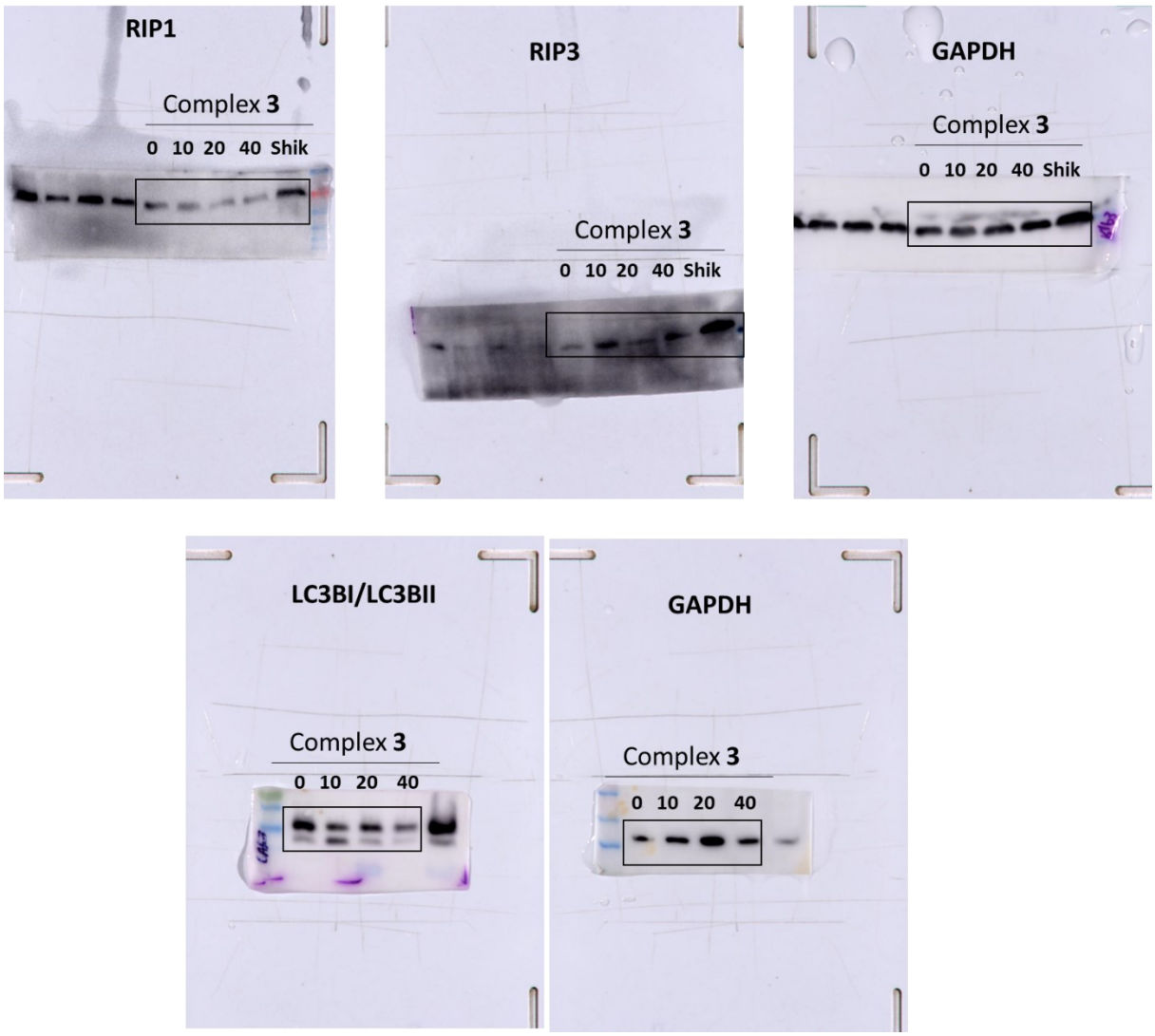


Figure S27. Uncropped full blots for Figures 11c and 11d.

Synergistic *in vitro* antitumor activity of complex 3 and cisplatin.

In a 96 well plate, 100 µL cell suspension of A549 cells at a density of 2000 cells per well were seeded and incubated for 20-24 h. The following day, fresh stock solution of complexes was prepared in DMSO (for complex **3**) or in water (for cisplatin), exact concentrations were determined by quantifying metals using ICP-MS and immediately serially diluted using appropriate complete culture medium. The cells were treated with the following concentrations of Complex **3** and cisplatin as follows: Complex **3** (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µM), Cisplatin (0, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µM), and Complex **3** combined with Cisplatin 1:1 (0.25 + 0.25, 0.5 + 0.5 , 1 + 1, 2 + 2, 4 + 4, 8 + 8, 16 + 16, 32 + 32 µM), 1:2(0.25 + 0.5, 0.5 + 1 , 1 + 2, 2 + 4, 4 +8, 8 + 16, 16 + 32 µM), 2:1 (0.5 + 0.25, 1 + 0.5 , 2 +1, 4 + 2, 8 + 4, 16 +8, 32 + 16 µM), 1:4 (0.25 + 1, 0.5 + 2 , 1 + 4, 2 + 8, 4 + 16, 8 + 32 µM), 4:1 (1 + 0.25, 2 + 0.5, 4 + 1, 8 + 2, 16 + 4, 32 + 8 µM), 1:8 (0.25 + 2, 0.5 + 4 , 1 + 8, 2 + 16, 4 + 32 µM), 8:1 (2 + 0.25,

4 + 0.5, 8 + 1, 16 + 2, 32 + 4 μM) for 48 h. The solutions were then added to attain a volume of 100 μL per well (total volume per well 200 μL, final DMSO concentration was ≤ 0.25 % in treated as well as untreated control wells). The plate was then incubated for desired periods, 48h or 72 h. Subsequently, the media was replaced with 200 μL fresh media containing 0.4 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and incubated further for 3h. The media was then aspirated and 180 μL/well of DMSO was added to lyse the cells and dissolve the resulting purple colored formazan crystals. The absorption was recorded at 570 nm using a BioTek Cytation 5 multi-well plate reader. The cell viability was calculated by normalizing each absorption value with respect to the untreated control and plotted against the applied concentrations using a Gen5 software to obtain the dose-response curve and IC_{50} values. The reported IC_{50} values are the average from at least three independent experiments, each of which consisted of either three or six replicates per concentration level.

Plasma stability.

Human plasma was purchased from Sigma Aldrich, India (H4522-100 mL). The experiments were performed as per guidelines from institutional biosafety committee (note: no special approval was required for small scale analytical experiments with this commercially available human plasma). 100 μL of human plasma and 100 μL of PBS were taken in a 1 mL vial. To this vial, 6 μL of 10 mM solution each of **3** and carbamazepine in DMSO were added. The mixture was shaken on a rotor at 37 °C for required period. Then 600 μL methanol was added to precipitate the proteinswhich were pelleted by centrifugation and 10 μL of the supernatant was directly injected into UPLC. The composition of the mixture at different time points was analysed to determine the percentage of intact compound **3**.

***In vivo* toxicity assessment in zebrafish model.**

Maintenance of zebrafish: Wild-type AB zebrafish (*Danio rerio*) was maintained in a state-of-the-art zebrafish housing system (Techniplast, Italy). The water's conductivity, pH, and temperature were maintained at ~500 μS/cm, ~7.5, and 28°C, respectively.

Statement of ethics: Less than 5 days post-fertilization (dpf), zebrafish embryos were used in this study. Experiments on zebrafish embryos were carried out following the Institutional Animal Ethics Committee (IAEC), ARI, and guidelines of the “Committee for the Purpose of Control and Supervision of Experiments on Animals” (CPCSEA), Government of India, recommendations.

Small molecule treatment and Brightfield Imaging: A 10 mM stock solution of complex **3** was prepared in DMSO. To obtain a 2.5, 4, 10, or 12 µM working concentrations of complex **3**, 10 mM stock solution was diluted in embryo water. To analyze the maximum safe concentration of the drug, twenty 26-hour post-fertilization (hpf) wild-type embryos were treated with 2.5/4/10 or 12 µM complex **3** or vehicle control (1.2 µL DMSO per mL of E3) and maintained in 10 ml embryo water at 28˚C until 72 hpf. To explore the effect of complex **3** on the embryonic development and viability of the treated embryos, brightfield images of the DMSO or complex **3**-treated embryos at 72 hpf were captured with the help of a Leica M205 FA stereoscope.

Statistical analysis: The embryo viability was examined using an unpaired t-test with Welch's correction. Data processing was carried out using Prism8 software. P values were computed following the mean ± SEM format of the data.

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2. D. Bornemann, L. Schlemper, N. Trapp and A. Togni, Expanding the Scope of Water-Stable Rhenium(V)-NHC Complexes – Synthesis, Characterization, and Derivatization, *Eur. J. Inorg. Chem.*, 2020, **2020**, 1004-1010.
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