

Supporting Information

De Novo Design of Thioredoxin Reductase-Targeted Heterometallic Titanocene–Gold Compounds of Chlorambucil for Mechanistic Insights in Renal Cancer

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1. Experimental

1.1. Materials and methods

All materials used were received from commercial sources unless stated otherwise. ^1H , ^{13}C NMR spectra were recorded on a 400 Mercury Plus Varian instrument operating at room temperature (400 MHz for ^1H and 100 MHz for ^{13}C relative to TMS, and 162 MHz relative to 85% H_3PO_4) at ambient temperature in $\text{DMSO-}d_6$. NMR annotations used: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, bs = broad singlet, m = multiplet. Elemental analyses were performed with an EA 3000 CHNS. ESI mass spectra were recorded on a Waters LCT Premier XE spectrometer.

1.2. Synthesis of the ligand Chlorambucil- alkynyl; 4-(4-(bis(2-chloroethyl)amino)phenyl)-N-(prop-2-yn-1-yl)butanamide (CHL-CCH)

To a solution of chlorambucil (0.304 g, 1 mmol) in DMF (10 mL) was added *N,N*-diisopropylethylamine (DIEA) (0.387 g, 3 mmol) and treated with *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC-HCl) (0.395 g, 2 mmol), 1-hydroxybenzotriazole hydrate ($\text{HOBT}\cdot x\text{H}_2\text{O}$) (0.270 g, 2 mmol) and propargylamine (96 μL , 1.5 mmol) at 0 °C. The reaction was stirred at room temperature for 12 h under nitrogen. Then, the mixture was diluted with water (10 mL) and extracted with dichloromethane (50 mL). The organic layer was dried with sodium sulfate (Na_2SO_4) and concentrated in vacuum. The resulting material was purified by silica gel column chromatography (mobile phase: petroleum ether/ethyl acetate 7:3) to provide the product as pale beige solids (0.250 g, 74% yield, 1 mmol). Anal. Calc. (%) for $\text{C}_{17}\text{H}_{22}\text{Cl}_2\text{N}_2\text{O}$ (340.1109): C, 59.83; H, 6.50; N, 8.21; Found (%): C, 59.80; H, 6.49; N, 8.19. TOF-MS: 341.1024 $[\text{M} + \text{H}]^+$. ^1H NMR (CDCl_3): δ 8.09 (s, 1H, H-c), 7.06 (d, 2H, H-6,10, $J = 7.6$ Hz), 6.86 (d, 2H, H-7,9, $J = 7.6$ Hz), 4.18 (d, 2H, H-b, $J = 7.6$ Hz), 3.60-3.69 (m, 8H, H-

11,12), 3.09 (s, 1H, H-a), 2.58 (t, 2H, H-4, $J = 7.6$ Hz), 2.41 (t, 2H, H-2, $J = 7.6$ Hz), 1.99-2.04 (m, 2H, H-3). ^{13}C NMR (CDCl_3): δ 174.7 (C-1), 144.1 (C-8), 134.0 (C-5), 128.8 (C-6,10), 113.6 (C-7,9), 81.2 (C-a or a'), 74.2 (C-a or a'), 53.1 (C-12), 42.8 (C-11), 34.9 (C-4), 32.1 (C-2), 28.2 (C-b), 26.7 (C-3).

1.3. Synthesis of complex $\text{K}[(\text{CHL-CC})\text{AuCl}]$, **1**

To a solution of ligand CHL-CCH (0.340 g, 1 mmol) in methanol (20 mL) was added KOH (0.561 g, 1 mmol) and stirred for 30 min. Then, chloro(dimethyl sulfide)gold(I), $(\text{CH}_3)_2\text{SAuCl}$, (0.294 g, 1 mmol) was added to the solution forming a suspension and dichloromethane (10 mL) was added dropwise to the solution until the solid was completely solubilized. The solution was stirred for 48 h under light protection. Then, the solution kept for 72 h at -20 °C. White crystals were formed during storage and were filtered off and washed 2 times with water (30 mL) and dried in vacuum (0.377 g, 62% yield, 1 mmol). Anal. Calc. (%) for $\text{C}_{17}\text{H}_{21}\text{AuCl}_3\text{KN}_2\text{O}$ (610.0022): C, 33.37; H, 3.46; N, 4.58; Found (%): C, 33.34; H, 3.44; N, 4.57. TOF-MS: 571.0982 [M - K] $^-$. ^1H NMR (CDCl_3): δ 8.01 (s, 1H, H-c), 6.92 (d, 2H, H-6,10, $J = 7.6$ Hz), 6.81 (d, 2H, H-7,9, $J = 7.6$ Hz), 4.19 (d, 2H, H-b, $J = 7.6$ Hz), 3.56-3.68 (m, 8H, H-11,12), 2.68 (t, 2H, H-4, $J = 7.6$ Hz), 2.48 (t, 2H, H-2, $J = 7.6$ Hz), 2.02-2.09 (m, 2H, H-3). ^{13}C NMR (CDCl_3): δ 174.9 (C-1), 144.9 (C-8), 134.0 (C-5), 129.1 (C-6,10), 114.2 (C-7,9), 73.0 (C-a or a'), 66.1 (C-a or a'), 53.9 (C-12), 43.2 (C-11), 35.0 (C-4), 33.1 (C-2), 28.9 (C-b), 27.7 (C-3).

1.4. Synthesis of complex $(\text{CHL-CC})\text{Au}(\mu^2\text{-}\eta^2\text{-CS}_3)\text{Ti}(\eta^5\text{-Cp})_2$, **2**

To the complex **1** (0.610 g, 1 mmol) in methanol (10 mL), AgNO_3 (0.17 g, 1 mmol) was added. After stirring for 4 h at room temperature under light protection, the white solid formed (AgCl) was filtered off. The filtrate solutions were added slowly to a methanolic solution (5 mL) of sodium trithiocarbonate (0.16 g, 1 mmol) and titanocene dichloride (Cp_2TiCl_2) (0.25 g, 1 mmol) in chloroform (5 mL) and stirred for 5 h under light protection and an inert atmosphere.

The solvent was removed in vacuum. The crude product was then purified via flash column chromatography on silica gel using hexane–dichloromethane (1: 1 v/v) as eluent. The heterometallic complex **2** was then isolated as orange solids (0.472 g, 57% yield, 1 mmol). Anal. Calc. (%) for $C_{28}H_{31}AuCl_2N_2OS_3Ti$ (822.0121): C, 40.84; H, 3.79; N, 3.40; Found (%): C, 40.81; H, 3.77; N, 3.38. TOF-MS: 823.0118 $[M + H]^+$. 1H NMR ($CDCl_3$): δ 8.06 (s, 1H, H-c), 6.97 (d, 2H, H-6,10, $J = 7.6$ Hz), 6.84 (d, 2H, H-7,9, $J = 7.6$ Hz), 6.64 (s, 10H, H-Cp), 4.21 (d, 2H, H-b, $J = 7.6$ Hz), 3.52-3.62 (m, 8H, H-11,12), 2.70 (t, 2H, H-4, $J = 7.6$ Hz), 2.51 (t, 2H, H-2, $J = 7.6$ Hz), 2.04-2.10 (m, 2H, H-3). ^{13}C NMR ($CDCl_3$): δ 252.4 (C-CS₃), 174.9 (C-1), 145.0 (C-8), 134.9 (C-5), 129.9 (C-6,10), 118.1 (C-Cp), 114.9 (C-7,9), 74.2 (C-a or a'), 66.9 (C-a or a'), 54.9 (C-12), 44.4 (C-11), 35.0 (C-4), 32.2 (C-2), 29.9 (C-b), 26.8 (C-3).

1.5. Stability tests

The stability of the compounds was tested by dissolving the compounds in PBS buffer/1% DMSO or DMEM (Dulbecco's Modified Eagle's Medium - high glucose) /1% DMSO solution and keeping them for 72 h at 37 °C. Briefly, a 10 μ L of the solution was injected into an HPLC system (Thermo, USA) connected to a UV/Vis spectrophotometer. A Hypersil Gold Dim (100 \times 2.1 mm, Thermo, USA) reversed-phase column was used at a flow rate of 0.5 mL min⁻¹. The mobile phase was 70:30 acetonitrile (0.1% trifluoroacetic acid): water (0.1% trifluoroacetic acid).

1.6. Cell culture

The human MCF-7, MDA-MB-231 breast cancer cell lines, A498, Caki-1 renal cancer cell lines, and MRC-5 non-tumorigenic cells (human lung fibroblasts) were obtained from the American Type Culture Collection (ATCC, USA). All reagents and cell culture media were purchased from Gibco Company (Germany). The cells were maintained in Dulbecco's Modified

Eagle Medium (DMEM) supplemented with 10% FBS, 100 IU mL⁻¹ of penicillin, 100 µg mL⁻¹ of streptomycin and 2 mM of Glutamax at 37 °C in a humidified incubator at 5% CO₂.

1.7. Cytotoxicity

The MCF-7, MDA-MB-231, A498, Caki-1, and MRC-5 were analyzed for viability post treatment using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following previously reported procedures.¹ The stock solutions of free ligands and the complexes **1**, **2**, auranofin, and also Cp₂TiCl₂ + complex **1** (1:1 equivalents) were prepared by dissolving the compounds in DMSO (0.1%) and for cisplatin by dissolving in 0.9% NaCl. The stocks were further diluted with the respective medium containing 10% FBS (0.02–10 µM) before addition to the cells. Cells were trypsinized with 0.25% trypsin-EDTA and counted with 0.4% trypan blue. The cells were seeded at a concentration of (3-10) × 10³ cells/well, dependent upon the growth characteristics of the cell line, and grown for 24 h at 37 °C in a humidified incubator. After 24 h, the medium was removed and replaced 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 10 µl of a 5 mg/mL MTT saline solution, and, following 5 h of incubation, 100 µl of a sodium dodecylsulfate solution in HCl (0.01 M) was added. The optical absorbance of each well (96-well plates) was quantified using EnVision multilabel plate readers (PerkinElmer, Waltham, MA, USA) at 570 nm wavelength. The mean absorbance for each drug dose was expressed as a percentage of the control, untreated well absorbance and plotted vs drug concentration. The IC₅₀ values were determined by nonlinear regression analysis using GraphPad Prism software. The IC₅₀ value was calculated as the concentration reducing the proliferation of the cells by 50% and is presented as a mean (±SE) of

three independent experiments each with triplicates. $P < 0.05$ was considered to be statistically significant.

1.8. Preparation of thioredoxin reductases

The cytosolic thioredoxin reductase (TrxR1) and mitochondrial thioredoxin reductase (TrxR2) from rat liver were obtained from Sigma-Aldrich and used without further purification.

1.9. In vitro TrxR1 and TrxR2 inhibition

The assay was performed in 0.2 M Na, K-phosphate buffer (pH 7.2) containing 2 mM EDTA, 0.25 mM NADPH (Nicotinamide adenine dinucleotide phosphate) and about 0.5-2 μg of TrxR protein. The reaction was initiated by the addition of 3 mM DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) to compounds (1 μM) and the increase of absorbance was monitored at 412 nm over 5 min at 25 °C. Enzyme activity was calculated taking into account that 1 mole of NADPH yields 2 moles of CNTP anion (reduced DTNB).

1.10. Thioredoxin reductase inhibition studies in Caki-1 cells

Thioredoxin reductase inhibition studies in Caki-1 cells were performed as previously reported.^{2,3} For thioredoxin reductase activity assays, the complete cell lysates was obtained from Caki-1 cells treated with gold complexes **1** and **2**, auranofin (at IC_{50} concentration) for 24 h. Control cells that were not treated with complexes were also prepared. Then, the cells were collected, washed three times in PBS, and centrifuged. The samples were lysed by douncing using scrapers and sheer force through syringe with a 34 gauge needle in assay buffer (Sigma-Aldrich, Thioredoxin Reductase Assay kit, CS0170) added to 1mM protease inhibitor cocktail (Sigma-Aldrich, P1860). Then the lysates were centrifuged for 15 min to isolate insoluble material. Protein concentration in the cellular extracts was determined using the Bradford Reagent (Sigma-Aldrich) and bovine serum albumin as standard. The soluble lysates were incubated for 20

minutes in assay buffer before adding DTNB (5,5'-dithiobis(2-nitrobenzoic) acid) and absorbance was monitored at 412 nm over 30 min at 25 °C. Lysates were tested in three independent experiments. TrxR activity was calculated as micro-mole reduced DTNB per minute per mg of total protein at 25°C and pH7.0. The percentage of activity inhibition refers to control cells.

1.11. Cellular uptake

Caki-1 cancer cells were seeded at a density of 10^6 cells/dish and maintained at 37 °C in a 5% CO₂ atmosphere. Cells were washed with phosphate-buffered saline (PBS), and fresh growth medium (5 mL) was added to each dish that freshly prepared complexes **1** and **2** (IC₅₀ concentration) were added to cells. The dishes were incubated at 37 °C under for a period of 24 h. The growth medium was removed and each dish was washed with PBS. Trypsin (0.25% in PBS) was added, and cells were harvested from the plate by using ethylenediaminetetraacetic acid (EDTA)-trypsin and centrifuged. Dishes were washed with PBS, and this was added to the cell suspension to ensure that all the cells were collected. The resulting suspension was centrifuged, and the supernatant was discarded. The cell pellet was washed with PBS (2 mL) and saline (0.9% NaCl, 99.999% pure, 2 mL), and the supernatant was discarded each time. The number of cells per dish was determined by scoring one dish out of each series of four. The Au or Ti content was determined by inductively coupled plasma mass spectrometry (ICP-MS), after digestion of the cells in concentrated HNO₃ (Tracepur, Merck) and dilution to 10.0 mL. All the measurements were done in triplicate.

1.12. Interactions with plasmid DNA (Mobility shift assay)

10 µL aliquots of pBR322 plasmid DNA ($20 \mu\text{g mL}^{-1}$) in a buffer (5 mM Tris/HCl, 50 mM NaClO₄, pH = 7.39) were incubated with molar ratios between 0.25 and 4.0 of the compounds at

37 °C for 20 h in the dark. Samples of free DNA and cisplatin–DNA adduct were prepared also as controls. After the incubation period, 2 µL of loading dye were added to the samples, and of these mixtures, only 7 µL were finally loaded into the gel. The samples were separated by electrophoresis in 1% (w/v) agarose gel for 1.5 h at 80 V in Tris-acetate/EDTA buffer (TAE). Afterwards, the gel was dyed for 30 min with a solution of GelRed Nucleic Acid stain.

1.13. ROS generation

2',7'-dichlorofluorescein diacetate (H₂DCFDA) (Thermo Fisher) was used to evaluate the intracellular ROS level in the form of cellular peroxides. This cell-permeant dye is hydrolyzed by intracellular esterases into its non-fluorescent form (DCFH) and is then oxidized by intracellular free radicals to produce a fluorescent product, namely dichlorofluorescein (DCF). Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells/well and were incubated for 24 h at 37 °C. The medium was removed and 100 µL of a 20 µM DCFH-DA solution in PBS was added to each well and incubated at 37 °C and 5% CO₂ for 60 min in darkness. Cells were washed twice with 100 µL PBS and then treated with 100 µL of 10 µM H₂O₂ in PBS (positive control), with PBS alone (untreated control) and a concentration range of each of the compounds and fluorescence was measured at different time intervals over 2 h. Reactive oxygen species levels were determined by measuring the fluorescence of oxidized DCFH-DA using a Jasco FP 6600 spectrofluorometer at an excitation wavelength of 485 nm and a corresponding emission wavelength of 525 nm. The fluorescence intensity was calculated by the determined fluorescence intensity minus the fluorescence intensity of the complex. The values obtained from three independent experiments and presented with their standard deviations.

1.14. Apoptosis assays on Caki-1 cells

Apoptosis assays were performed by using cisplatin, complexes **1** and **2** (IC₅₀ concentrations) in Caki-1 cells as previously reported. ⁴

1.15. Density functional theory: Molecular structure and frontier molecular orbitals

Initial geometries of complexes **1** and **2** were modelled with GaussView 5.0. The geometries of the two complexes were optimized using the M06/6-31G+(d,p)//SDD model that specifies the use of Minnesota, M06 functional and mixed basis sets of 6-31G+(d,p) for C, H, N, S, O, and Cl atoms, and Stuttgart-Dresden (SDD) effective core pseudopotential (ECP) on Au and Ti. Placement of SDD on transition metals has yielded good results in previous studies, ^{5, 6} while documented evidences also abound where M06 functional had been successfully used in DFT calculations. ⁷ Optimized geometries were verified to be the actual minimum energy structures without negative vibrational frequencies. Gaussian 09 software was utilized for the calculations. ⁸ Optimized structures and frontier molecular orbitals (FMOs) canonical isosurfaces were visualized with GaussView 5.0. Selected reactivity indices were derived from the FMO energies of the optimized structures. Energies of the highest occupied and lowest unoccupied molecular orbitals (E_{HOMO} and E_{LUMO} respectively) were evaluated in electron volts (eV), while the energy gap (ΔE) and electronegativity were calculated as reported elsewhere. ⁹

1.16. Molecular docking of complexes 1 and 2 with thioredoxin reductase and cytosolic DNA

1.16.1 Homology modelling and MD simulation of human Trx2 (mitochondrial)

3D structure of human TrxR was modeled using software Modeller 9.20. Homologous proteins were searched using human TrxR protein sequence (NCBI: NP_006431.2) as the query sequence within protein data bank (PDB) by Position-Specific Iterated Basic Local Alignment Search Tool (PSI-BLAST). Top ranked identical proteins include 1ZDL, 3DGZ, 2NVK, 3DH9, 3DGH and

3EAN. 1ZDL was used as the template for the homology modeling since it has the highest total score from the PSI-BLAST (sequence homology of 85%). A 3D structure having amino acid sequence from 1 to 524 was modeled according to the sequence alignment of human Trx2 and 1ZDL sequence (Figure S20b). Residue SeCys520 was mutant to Cys520 during the modeling process because residue SeCys is a non-standard residue which cannot be recognized by any current software.

The modeled protein was optimized by molecular dynamics simulation in a box of water using the OPLS all-atom force field and SPCE water model on a 2fs timescale and a total time of 1ns using a leap-frog integrator. The MD simulation was done on a GROMACS engine, version 2018.3, installed on a macintosh machine. The simulated protein was extracted in a pdb format with VMD and a stereochemical assessment was done with a RAMACHANDRAN plot on the RAMPAGE server.¹⁰ The simulated protein was aligned with the base homology model and template and the RMSD was estimated using pymol (Figure S20).

1.16.2 Molecular Docking of ctDNA human Trx1 and Trx2 with synthesized complexes

The optimized structure of the Trx2 and downloaded crystal structure of Trx1 (PDB ID = 3QFA) were prepared for docking using the MGL Tools suite from Scripps Research Institute, USA. The Gasteiger charges were computed, all hydrogens except polar hydrogens removed and the grid box size was chosen to cover most of the protein (126 points in the XYZ direction). The box was centred at 92.1, 21.8, -85.8 for the Trx1 and 50.8, 50.6, 47.6 for Trx2 which is the reference point in the protein to which the cofactors are bound. The ground-state molecular structures of complex **1** and complex **2** derived from the DFT calculations were adopted for the molecular docking. The compounds (i.e. complex **1** and complex **2**) were docked with the prepared protein using AutoDock Tools-1.5.6, computing the binding energy of the ligands for

the protein and their inhibition constants. The output docking files were viewed using the MacPyMOLX11Hybrid which was also used to estimate the bond distances of the polar interactions.

Results

Synthesis and characterization

The purity of the synthesized compounds was considered by elemental analysis and high performance liquid chromatography (HPLC). Elemental analyses of tested compounds concurred with the expected values. Formation of complexes **1** and **2** were clearly confirmed by the absence of the terminal hydrogen signal of the ligand CHL-CCH in ^1H NMR spectra (Figures S1-S3). This peak appears at 3.09 ppm as singlet in ^1H NMR spectrum of free ligand. Also, the hydrogen signals of Cp rings of titanocene centered as singlet at 6.64 ppm in ^1H NMR spectrum of complex **2**. Moreover, the carbon of trithiocarbonate appeared at 252.4 ppm in ^{13}C NMR spectrum of complex **2**. ¹¹ These results confirmed the structure of complex **2** (scheme S1). The ESI-TOF MS spectra of ligand CHL-CCH, complexes **1** and **2** showed peaks centered at $m/z = 341.10$ ($[\text{M} + \text{H}]^+$), 571.09 ($[\text{M} - \text{K}]^-$), and 823.01 ($[\text{M} + \text{H}]^+$), respectively.

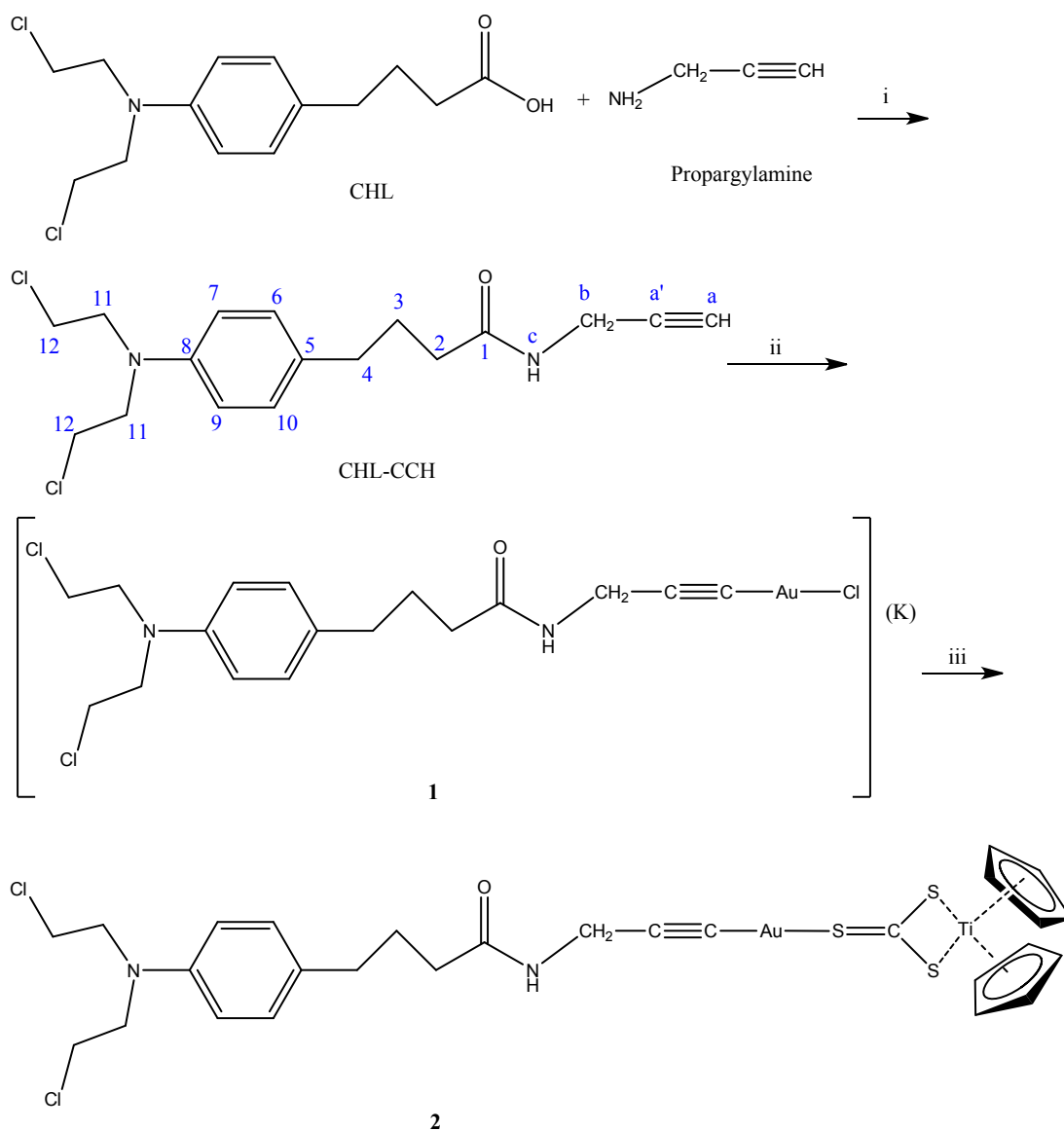
Stability of the compounds

The stabilities of the free ligand CHL-CCH, complexes **1** and **2** were evaluated in DMSO: PBS buffer (1:99 v/v) solution by HPLC-UV at 37 °C. The results confirmed the purity of the compounds and their stabilities during 72 h (Figure S10). Also, the stability of compounds were studied in DMEM (Dulbecco's Modified Eagle's Medium - high glucose) /1% DMSO solution during 72 h (Figure S11). For comparison, the HPLC of Cp_2TiCl_2 and mixing Cp_2TiCl_2 + complex **1** (1: 1 eq) in DMEM /1% DMSO solution were prepared (Figure S12). As it is clear,

there is no detectable change in retention time during 3 days, confirming the stability of these compounds under physiological conditions.

In vitro TrxR1 and TrxR2 inhibition

Complexes **1** and **2** were tested in vitro for their ability to inhibit both cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductases. Complex **2** showed IC₅₀ values at nanomolar concentrations as inhibitor to both cytosolic (TrxR1) and mitochondrial (TrxR2) with better activity than auranofin. Mitochondrial thioredoxin reductase (TrxR2) appeared less sensitive to the inhibitory effect of complex **2** compared to TrxR1 (Table S1). Complex **1** is more active towards TrxR1 than TrxR2, and showed better activity than auranofin against TrxR1 and the same activity as auranofin against TrxR2. Heteronuclear complex **2** is also more active than complex **1** against both TrxR1 and TrxR2.



Scheme S1. Synthetic pathways of the ligand CHL-CCH, the complexes **1** and **2**. Reagents and conditions: (i) EDC, HOBt, DIEA, DMF, 0 °C to RT, 12 h, 74%; (ii) $(\text{CH}_3)_2\text{SAuCl}$, KOH/MeOH/ CH_2Cl_2 , RT, 48 h, 62%; (iii) (a) AgNO_3 , 4h, RT (b) titanocene dichloride (Cp_2TiCl_2), sodium trithiocarbonate (Na_2CS_3), MeOH/ CHCl_3 , RT, 5 h, 57%.

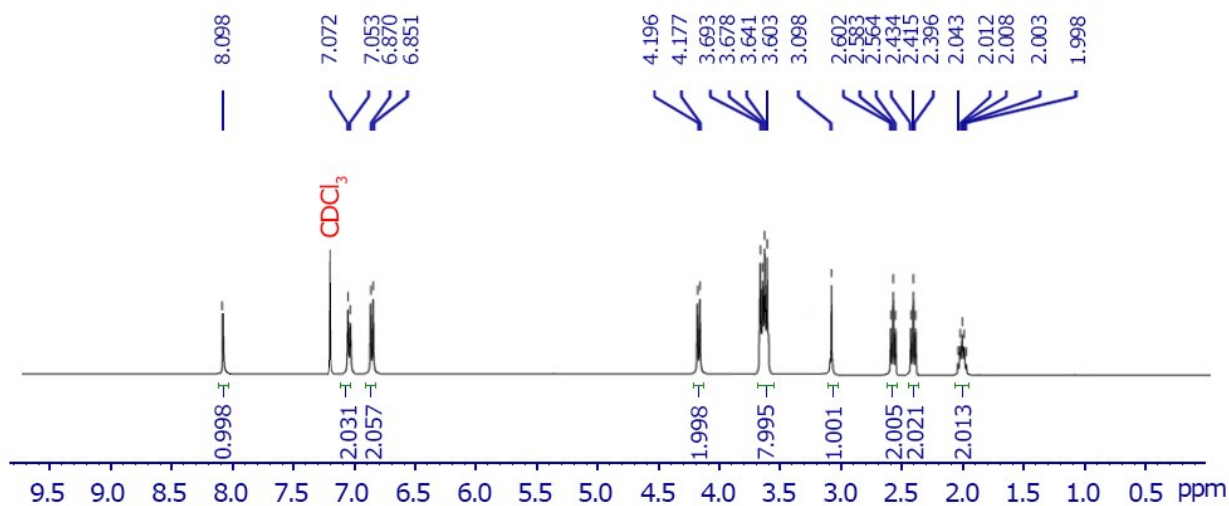


Figure S1. ¹H NMR spectrum of ligand **CHL-CC** (CDCl₃).

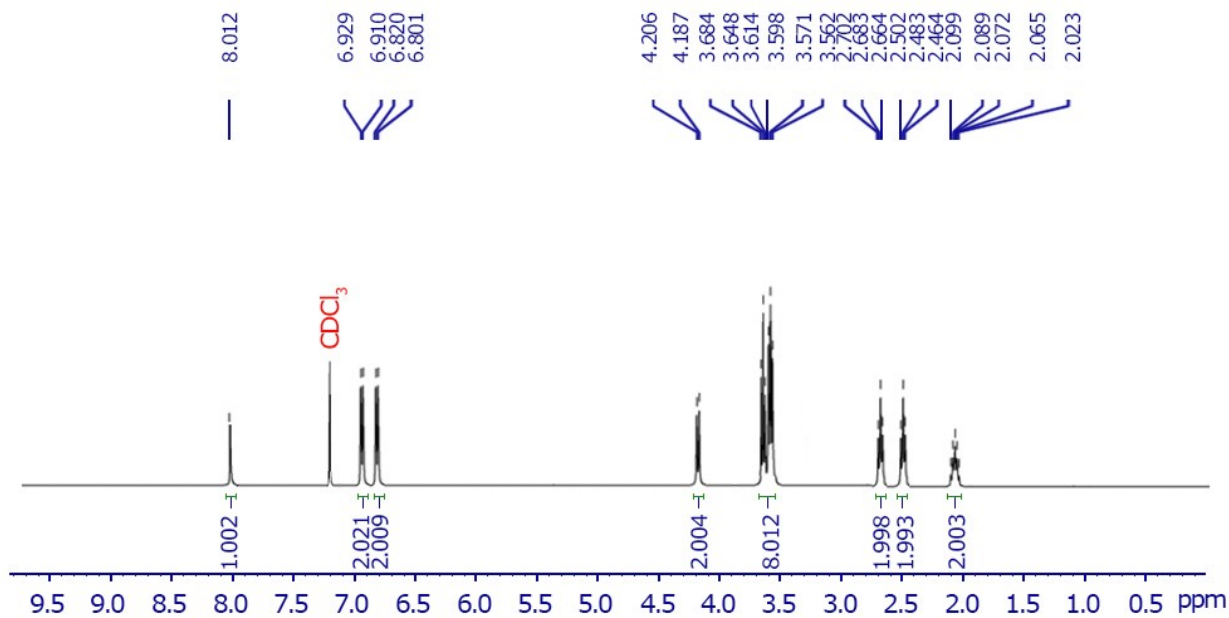


Figure S2. ¹H NMR spectrum of complex **1** (CDCl₃).

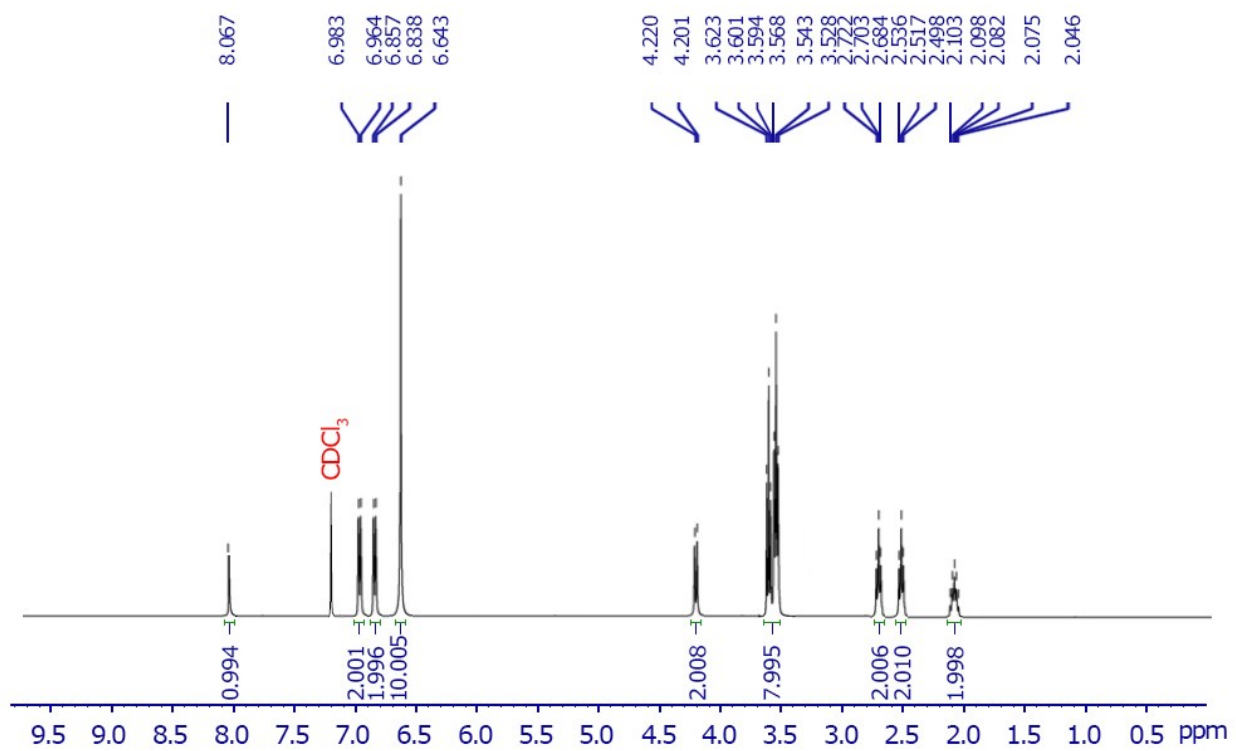


Figure S3. ¹H NMR spectrum of complex 2 (CDCl₃).

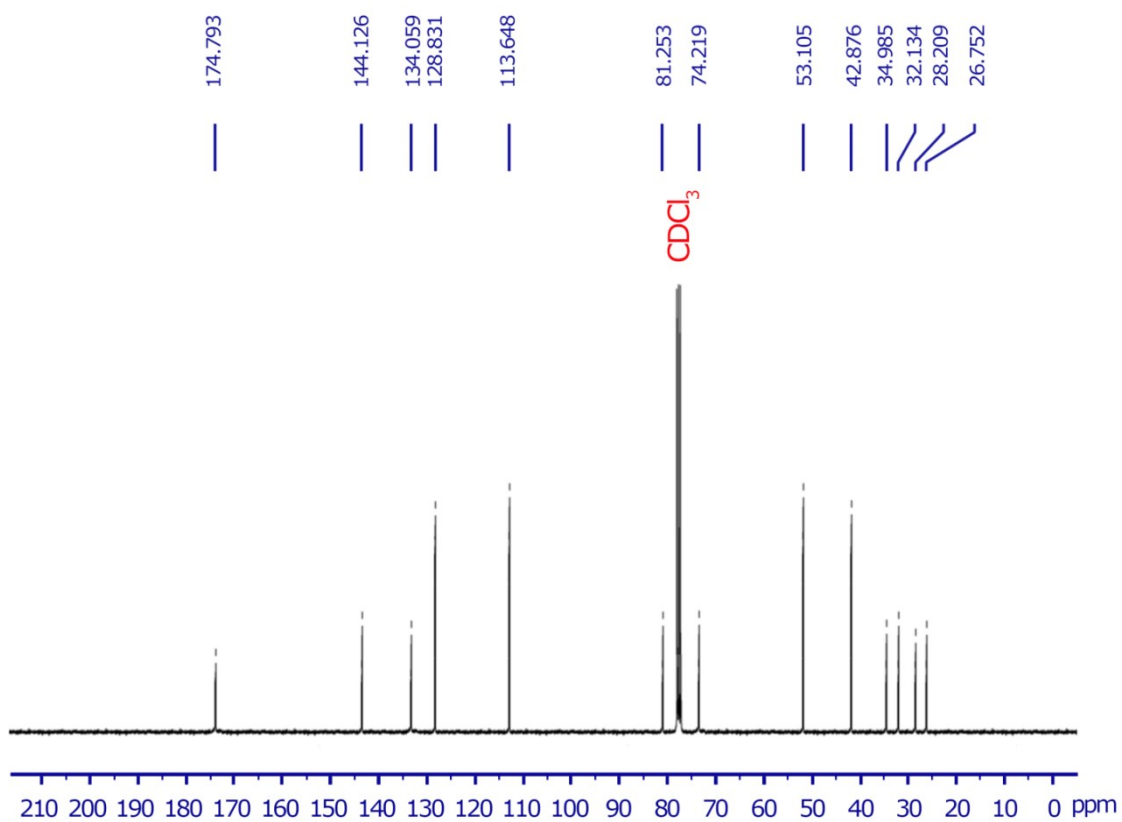


Figure S4. ¹³C NMR spectrum of ligand CHL-CC (CDCl₃).

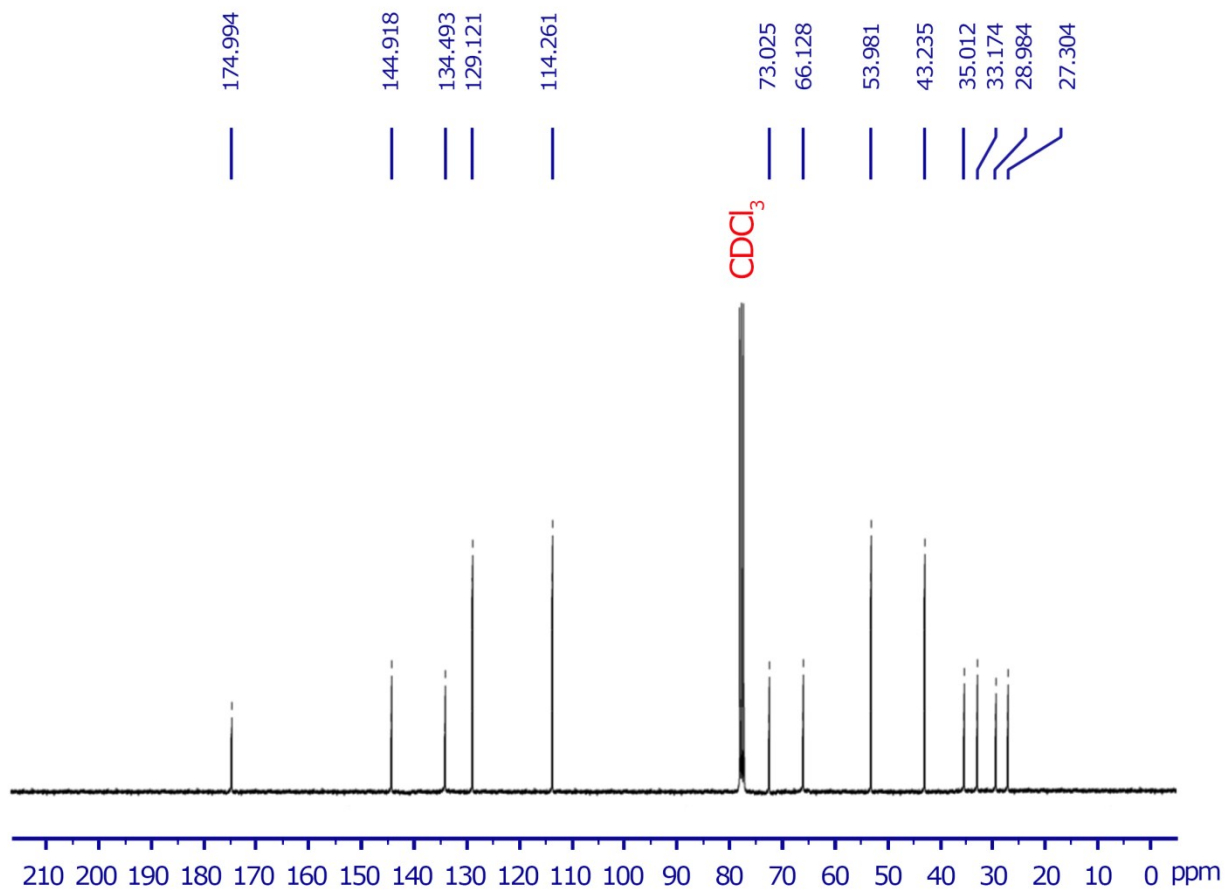


Figure S5. ¹³C NMR spectrum of complex 1 (CDCl₃).

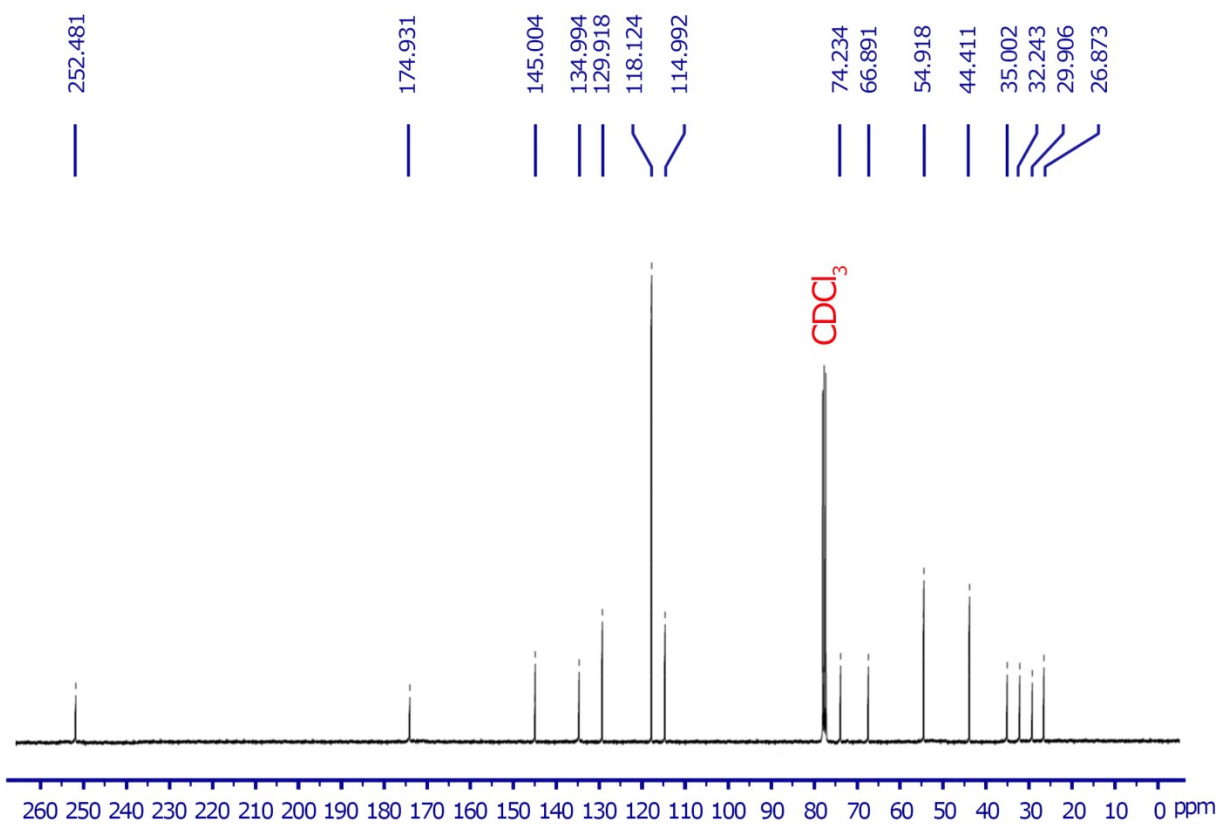


Figure S6. ¹³C NMR spectrum of complex 2 (CDCl₃).

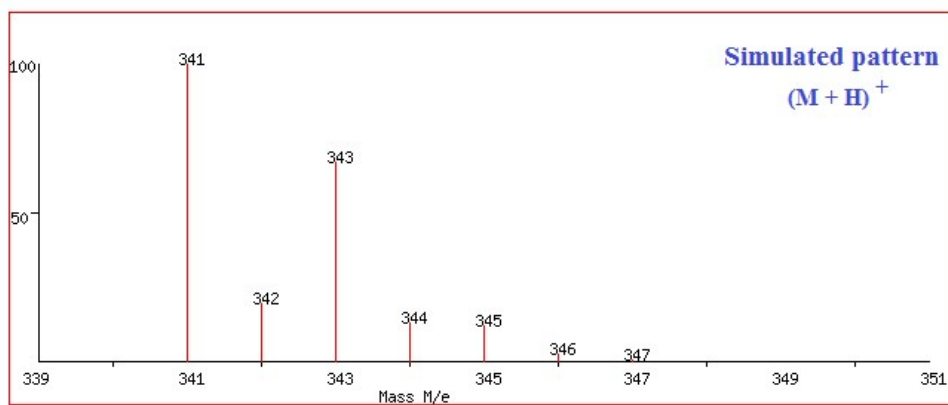
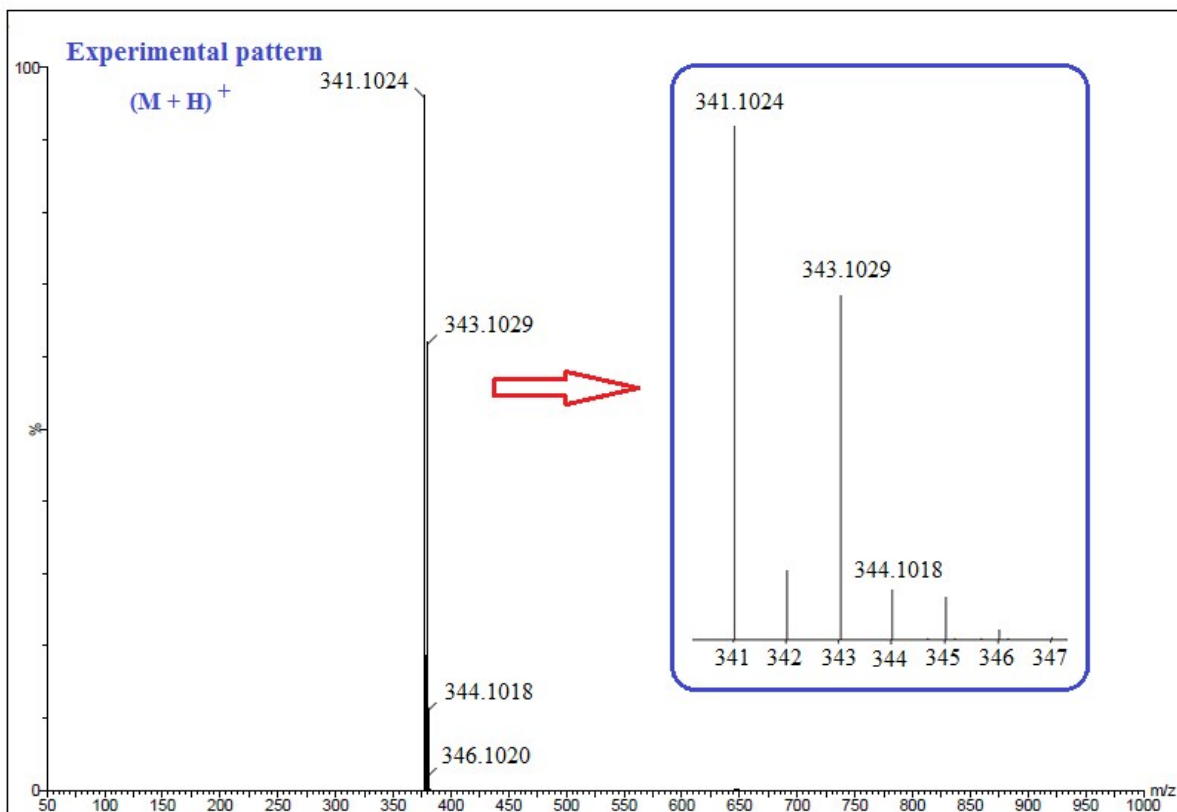


Figure S7. TOF MS spectrum of ligand CHL-CC.

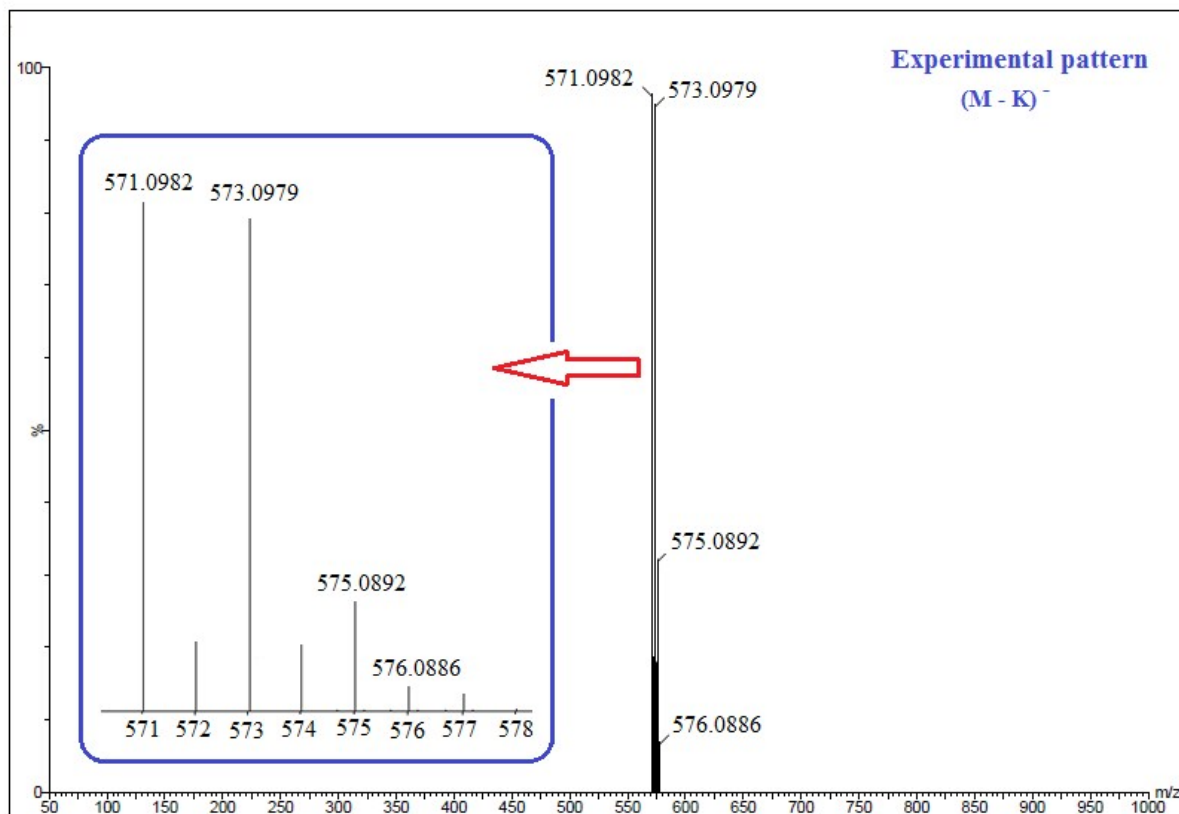


Figure S8. TOF MS spectrum of complex 1.

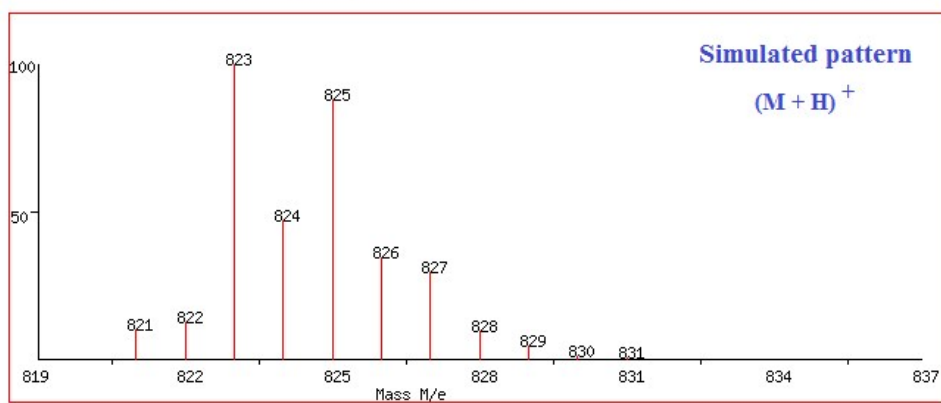
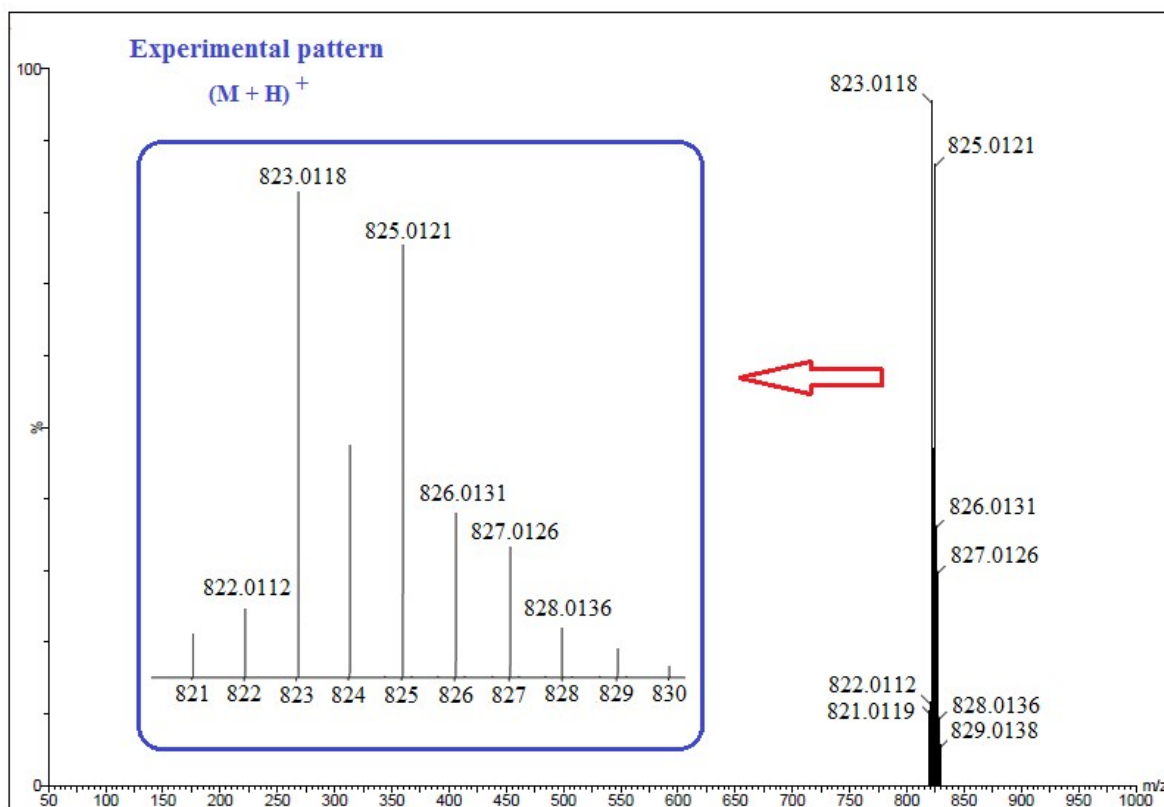


Figure S9. TOF MS spectrum of complex 2.

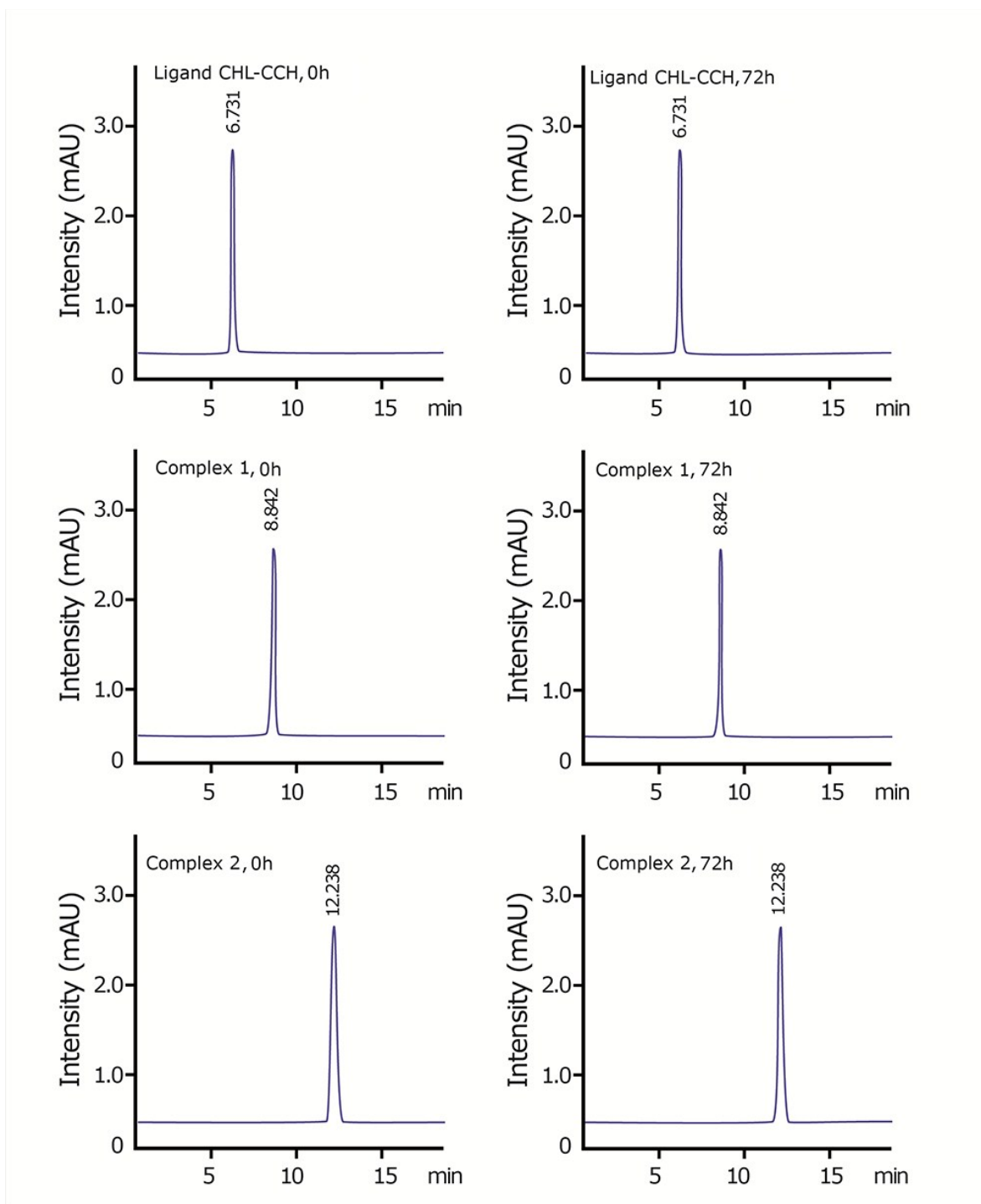


Figure S10. HPLC of the free ligand CHL-CC and complexes **1** and **2** in freshly prepared PBS buffer/1% DMSO solution recorded during 72 h of incubation time at 37 °C.

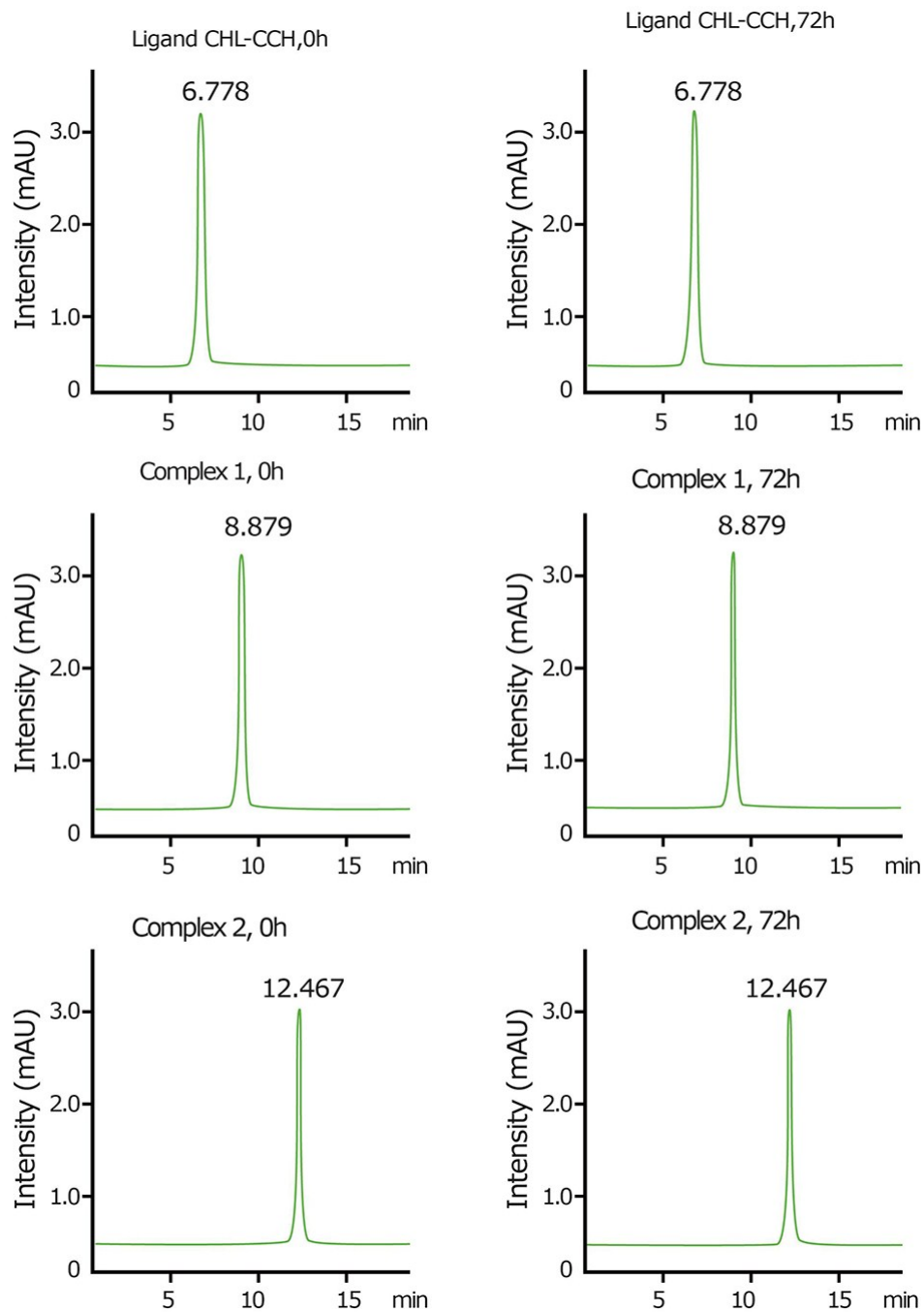


Figure S11. HPLC of the free ligand CHL-CC and complexes **1** and **2** in DMEM (Dulbecco's Modified Eagle's Medium - high glucose) /1% DMSO solution (0 h) and recorded after 72 h of incubation time at 37 °C.

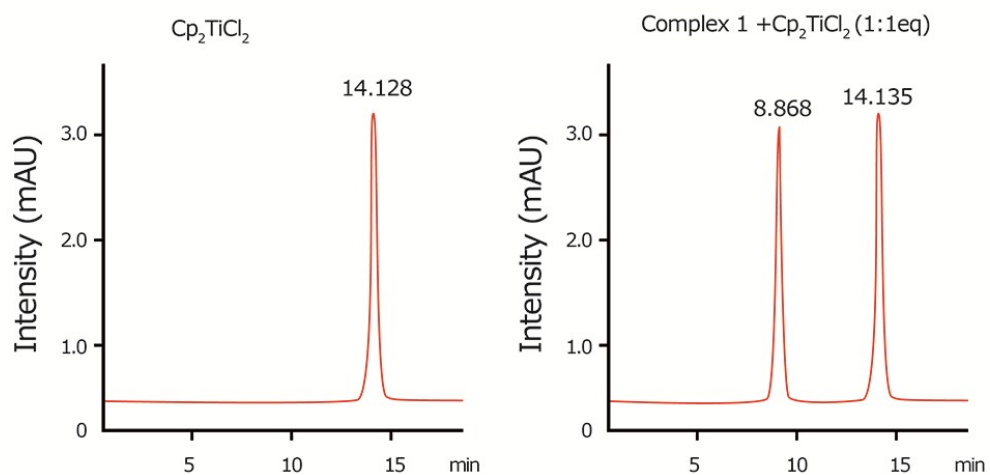


Figure S12. HPLC of Cp₂TiCl₂ and mixing Cp₂TiCl₂ + complex 1 (1: 1 eq) in DMEM (Dulbecco's Modified Eagle's Medium - high glucose) /1% DMSO solution.

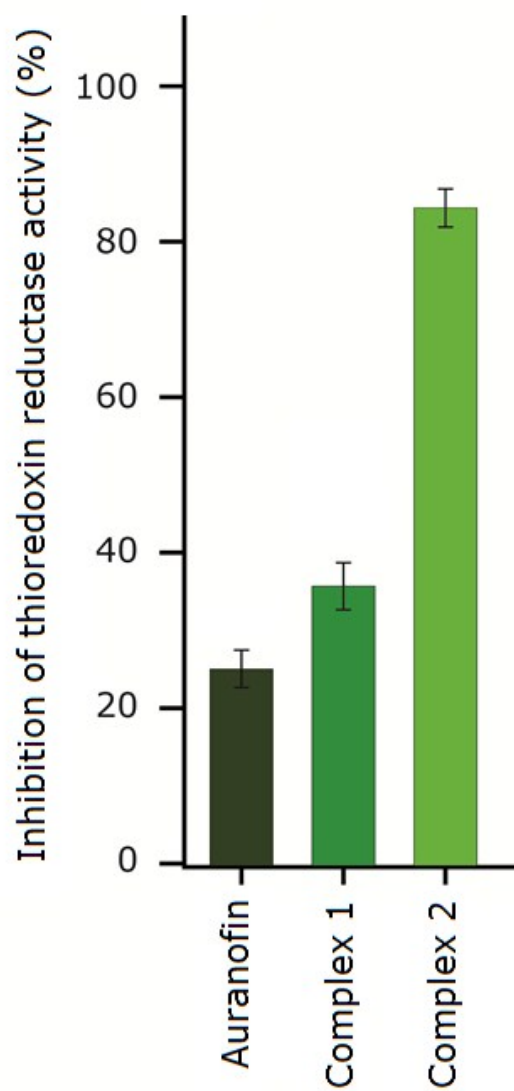


Figure S13. Inhibitory effects on TrxR activity in Caki-1 cells after 24 h incubation with complexes **1** and **2** and auranofin (IC_{50} concentration) and monitoring the lipoate reduction colorimetrically after 30 min incubation.

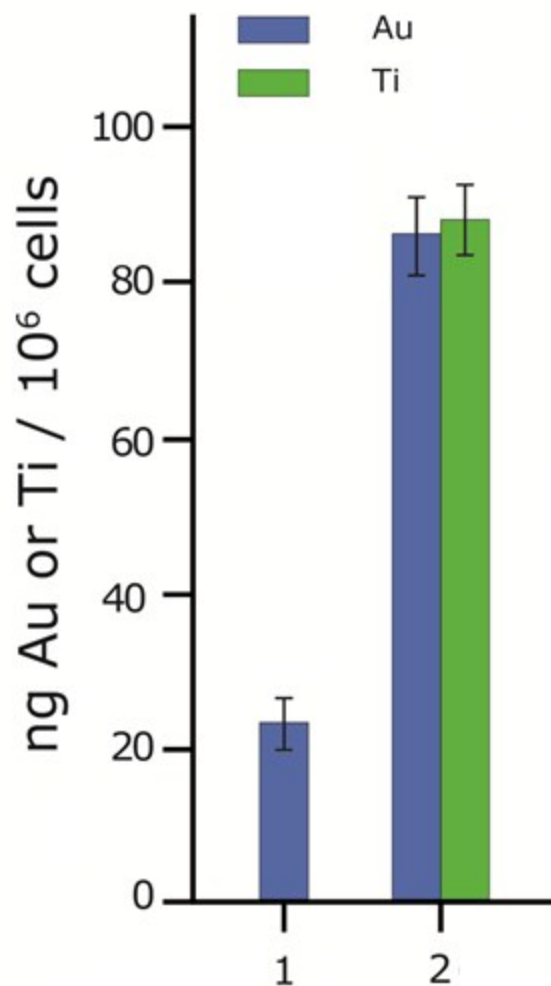


Figure S14. Intracellular Au or Ti accumulation in Caki-1 cells after 24 h incubation with complexes **1** and **2** (IC_{50} concentration). Experiments were performed in triplicate. Error bars indicate the standard deviation.

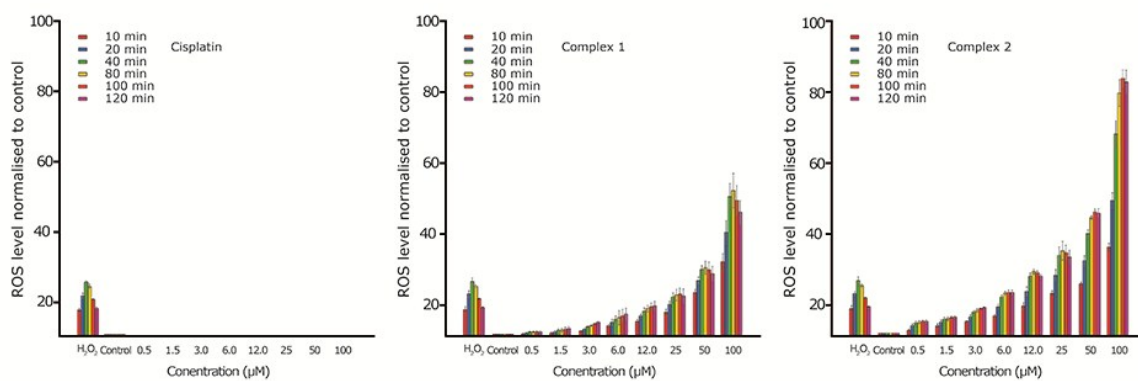


Figure S15. Time-course generation of the reactive oxygen species (ROS) within the cancer cell line Caki-1 after exposure to cisplatin, complexes 1, and 2 during 120 min.

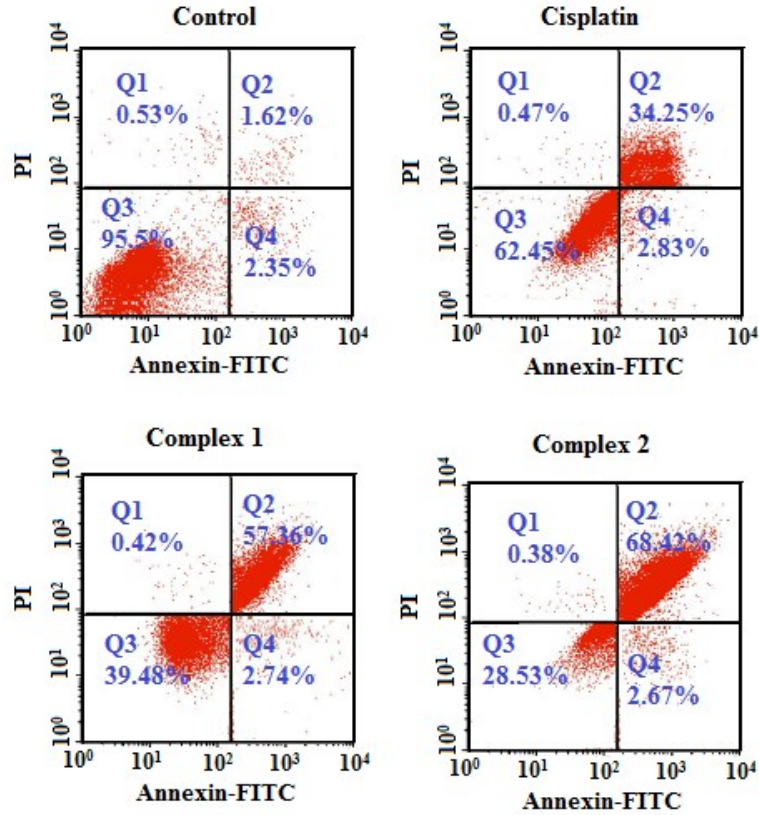


Figure S16. Flow-cytometric analysis of Caki-1 cells after staining with Annexin-V FITC and propidium iodide (PI) for 24 h of incubation with IC₅₀ (Top right quadrant, dead cells in late stage of apoptosis; bottom right quadrant, cells undergoing apoptosis; bottom left quadrant, viable cells). Representative data from three independent experiments are shown.

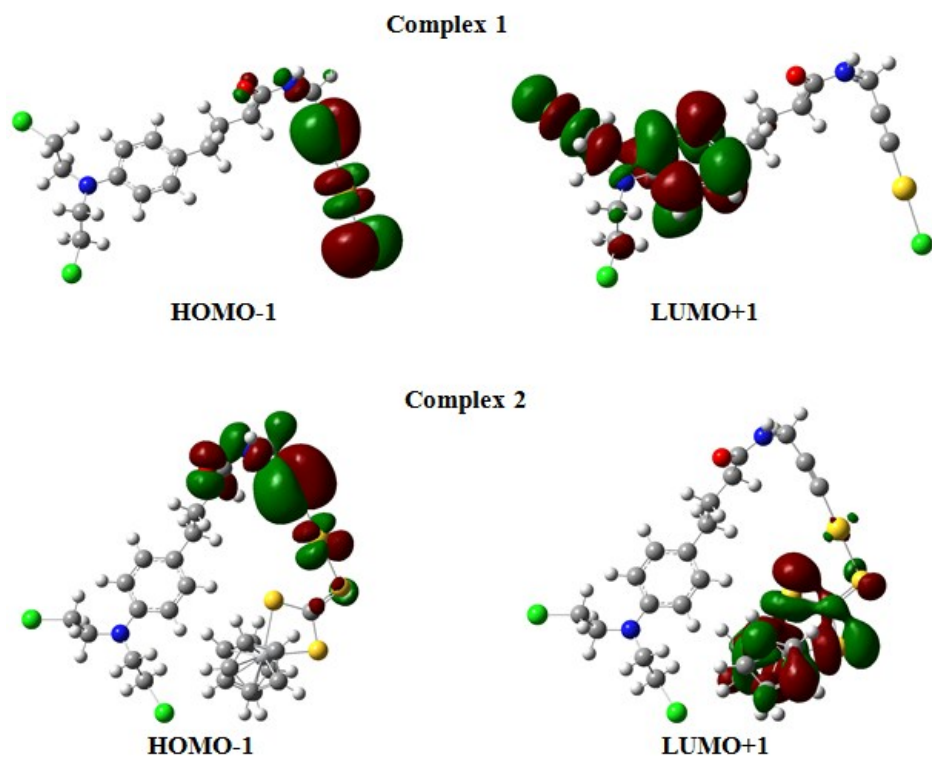
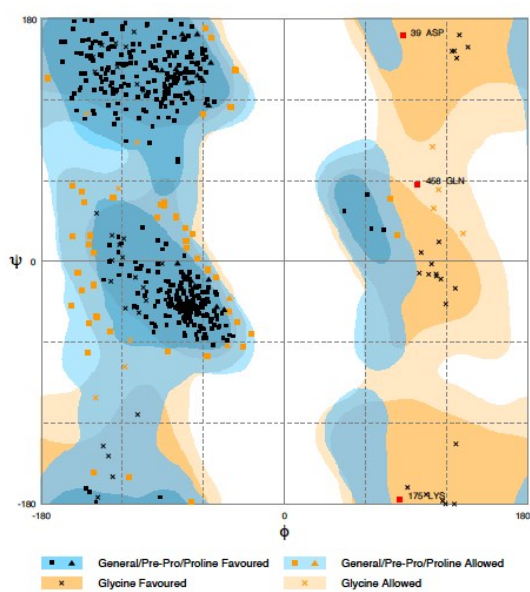
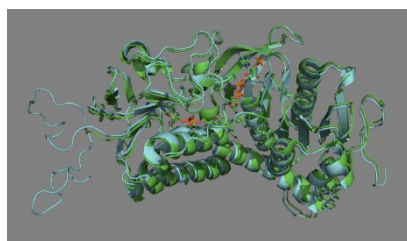


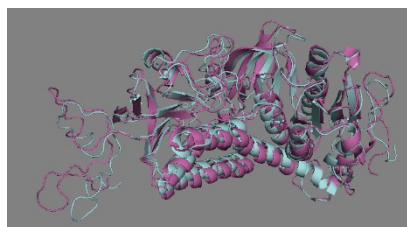
Figure S19. Canonical isosurfaces of HOMO-1 and LUMO+1 of complex 1 and complex 2.



(a)



(b)



(c)

Figure S20. (a) Ramachandran plot of the optimized (by MD simulation) model of Trx2, residues in allowed and favoured regions amount to 99.4% of the entire residues. (b) Superposition of the homology model (cyan) on the template (1ZDL, green), RMSD is 0.27. (c) Superposition of the optimized model (magenta) on the homology model (cyan), RMSD is 2.63.

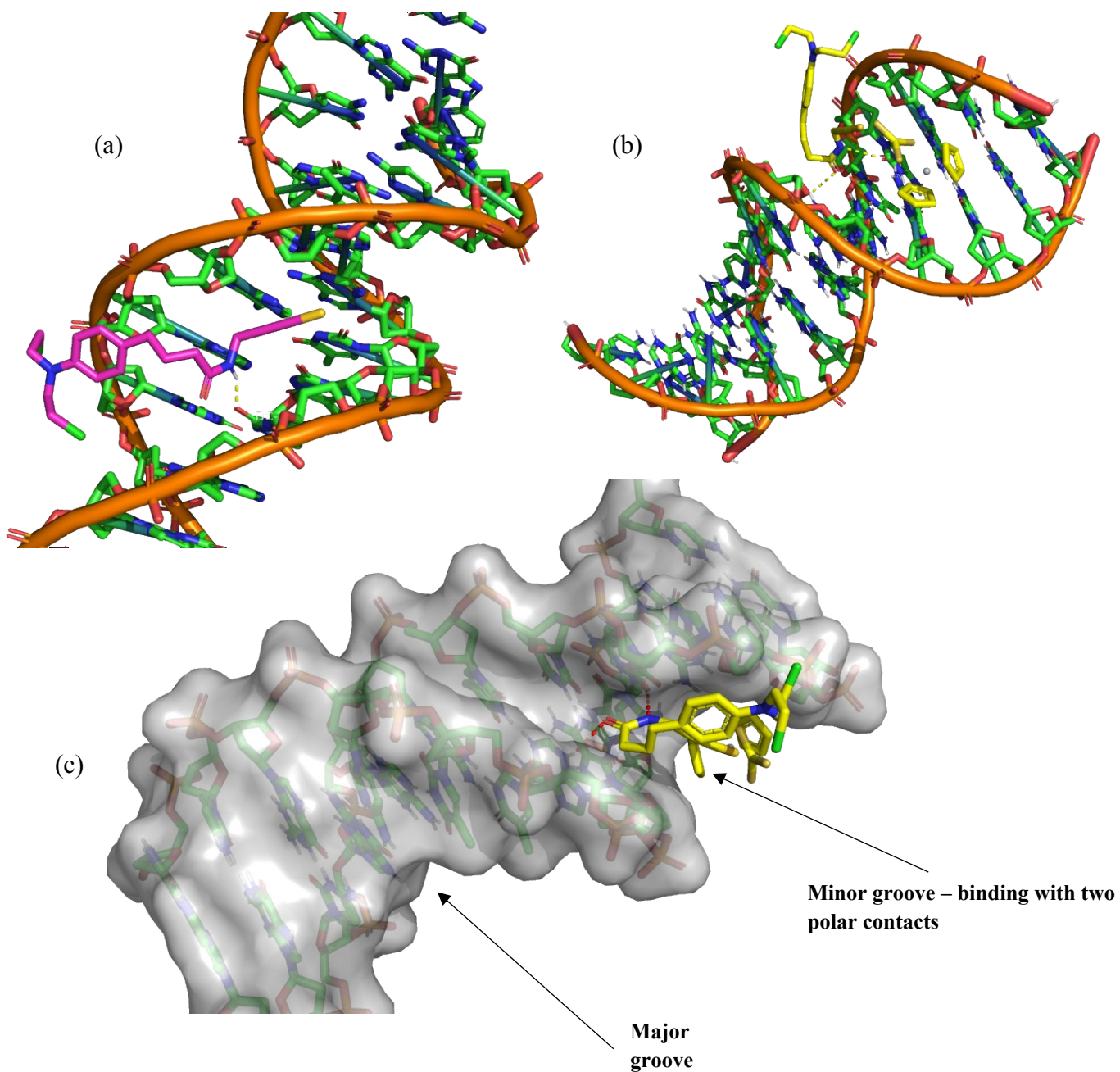


Figure S21. Stick rendering of DNA showing both synthesized complexes bound to different segments within the minor groove (a) and (b) for complexes **1** and **2** respectively. (c) is a surface rendering of the DNA showing complex **2** bound to the minor groove with two polar contacts.

Table S1. Cytotoxic activity and TrxR1 and TrxR2 inhibition of compounds.

Compounds	IC ₅₀ (μM) ± SD (72h)					SI ^a	IC ₅₀ (nM) ^b	
	MCF-7	MDA-MB-231	A498	Caki-1	MRC-5		TrxR1	TrxR2
CHL-CCH	> 50	> 50	> 50	> 50	ND	ND	-	-
Complex 1	> 50	> 50	2.36 ± 0.06	1.17 ± 0.05	1.86 ± 0.05	1.05	0.72 ± 0.05	3.21 ± 0.04
Complex 2	> 50	> 50	0.057 ± 0.01	0.016 ± 0.01	2.61 ± 0.08	71.5	0.18 ± 0.02	2.38 ± 0.05
Cp ₂ TiCl ₂	-	-	> 100	> 100	-	-	-	-
Cp ₂ TiCl ₂ + complex 1 (1eq:1eq)	-	-	3.35 ± 0.12	2.24 ± 0.10	-	-	-	-
Cisplatin	2.83 ± 0.15	3.78 ± 0.10	33.45 ± 0.16	21.68 ± 0.27	7.31 ± 0.10	0.26	-	-
Auranofin	-	-	3.85 ± 0.25	3.21 ± 0.40	-	-	1.09 ± 0.05	3.54 ± 0.07

ND: not determined. ^a SI was calculated by dividing the average IC₅₀ for normal cells MRC-5 by average IC₅₀ for A498 and Caki-1 cancer cells. ^b Thioredoxin reductase activity was assayed by measuring NADPH-dependent reduction of DTNB at 412 nm; IC₅₀ values were calculated by probit analysis (P < 0.05, x2 test).

Table S2. Interacting Residues/Bases and polar contacts

Compounds complexed with biomolecule	Interacting residues	Polar interactions (Å)
Complex 2 with Trx2	ARG193, VAL224, GLY225, ALA226, SER227, TYR228, MET247, MET248, ARG249, SER250, ARG254, GLY255, ALA280, PRO281, ILE316, GLY317, LYS340, ASP359, GLY363, ARG364, PRO365,	No polar contact
Complex 1 with Trx2	TYR228, GLY255, PHE256, ASP393, ASN394, VAL395, THR397, GLY409, LEU410	No polar contact
Complex 2 with Trx1	GLY197, ALA198, SER199, TYR200, ARG221, SER222, ARG226, GLY227, PHE228, THR274, ALA290, ILE291, GLY292, VAL340, GLU341, LEU342, THR372	Two polar contacts: Amide -CO of Ligand with -NH ALA198 (backbone), 2.1Å Amide -CO of ligand with -NH SER199 (backbone), 2.5Å
Complex 1 with Trx1	VAL62, GLY63, CYS64, PHE184, TYR200, LEU342, THR343, PRO344, PRO371, THR372, THR373, GLY446, GLU447, THR449, GLN450	One polar contact Amide -CO of ligand with -NH THR343 (backbone), 3.0 Å
Complex 2 with DNA	C3, G4, A5, A6, T7, T20, C21, G22, C23, G24	A6 (-NH) with amide -CO of Ligand, 2.6Å C21 (-CO) with amide -NH of ligand, 3.1Å
Complex 1 with DNA	G4, A5, A6, T7, T20, C21, G22, C23	C21 (-CO) with amide -NH of ligand, 1.7 Å

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