Combining a Ru(II)-arene complex with a NO-releasing nitrate-ester ligand generates cytotoxic activity

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Supporting Information

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Synthetic methods

Materials and characterization

$RuCl_3 \cdot H_2O$ (Pressure Chemical), 4-pyridinemethanol (TCI America), *N*-methyl-D-glucamine (Sigma-Aldrich), sulfuric acid (Anachemia), nitric acid (EMD), human serum (Sigma-Aldrich), and glutathione (Sigma-Aldrich) were used as purchased. Absolute methanol, absolute ethanol, absolute ether, and dichloromethane were all analytical grade and were used directly without further purification. *N*-Methyl-D-glucamine dithiocarbamate (MGD)[1] and $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ (**2**) were prepared according to previous reports.[2] Infrared spectra were recorded on a Thermo Nicolet Nexus 670 FTIR spectrometer equipped with a Pike MIRacle attenuated total reflection (ATR) sampling accessory (germanium crystal: 4000-700 cm^{-1}).

4-nitrooxymethylpyridine nitrate **(1)**

To concentrated nitric acid (68-70%, 4 mL) at 5 °C, 4-pyridinemethanol (2.3 g, 21.1 mmol) was added slowly, followed by concentrated sulfuric acid (95-98%, 4 mL), while maintaining the temperature of the reaction mixture below 10 °C. The mixture was stirred for 3 hours at 5 °C, and then poured into an ice-water mixture (40 mL). The pH of the mixture was adjusted to 7 with sodium hydroxide, and the mixture was then extracted with ethyl acetate (3×30 mL). Nitric acid (68-70%, 0.5 mL) was added to afford a white precipitate. The white solid was filtered off and used without further purification. Yield, 65%; melting point, 78-80 °C; $C_6H_7N_3O_6$ Calc. C, 33.19; H, 3.25; N, 19.34. Found C 33.57; H 3.42; N 18.94. IR(ν): 3105, 3076, 2941, 1644, 1407, 1283, 884 cm^{-1}. ^1H NMR (500 MHz, D_2O): δ 8.82 (d, J = 6.2 Hz, 2H), 8.12 (d, J = 6.1 Hz, 2H), 5.87 (s, 2H). ^{13}C NMR (126 MHz, D_2O): δ 154.84, 141.46, 125.04, 71.08. ESI(+)-MS m/z = 155.0452 $[4\text{-(nitrooxymethyl)pyridine}]^+$.

[(η⁶-p-cymene)Ru(4-nitrooxymethylpyridine)Cl₂] **(3)**

To a suspension of $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ (0.2312 g, 0.377 mmol) in methanol (15 mL), 4-(nitrooxymethyl)pyridine nitrate (**1**, 0.1753 g, 0.872 mmol) was added, and stirred at room temperature for 24 hours, producing a dark red solution. Diethyl ether (150 mL) was added to the reaction solution to produce a mixture of an orange precipitate and excess white **1**. The solids were isolated by gravity filtration and then added to a minimum volume of chloroform to dissolve the orange precipitate. **1** is insoluble in chloroform and was separated from the solution by gravity filtration. Diethyl ether (15 mL) was added to the orange filtrate and the mixture was stored at −18 °C overnight, which gave crystals of **3** co-crystallized with chloroform suitable for X-ray diffraction. To remove co-crystallized chloroform, **3** was dissolved in a minimal amount of acetone, and then solvent was removed under reduced pressure and the resulting orange powder was left under vacuum for two days. Yield, 46.5%; melting point, 131-133 °C; $C_{16}H_{20}Cl_2N_2O_3Ru$, Calc. C, 41.75; H, 4.38; N, 6.09. Found C, 41.92; H, 4.45; N, 6.42. IR (ν): 3058, 2964, 1632, 1422, 1282, 849 cm^{-1}. ^1H NMR (500 MHz, Chloroform-*d*): δ 9.10 (m, 2H), 7.31 (d, J = 6.0 Hz, 2H), 5.47 (s, 2H), 5.45 (d, J = 5.9 Hz, 2H), 5.23 (d, J = 5.9 Hz, 2H), 3.01 (m, J = 6.9 Hz, 1H), 2.12 (s, 3H), 1.32 (d, J = 6.9 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 155.35,

144.25, 122.93, 103.86, 97.32, 82.84, 82.41, 71.36, 30.81, 22.40, 18.39. ESI(+)-MS m/z $[M\text{-}Cl]^+$ = 425.018

Experimental procedures

Crystallographic structure determination

Single-crystal X-ray crystallographic analysis was performed on a Bruker SMART diffractometer equipped with an APEX II CCD area detector fixed at a distance of 5.0 cm from the crystal and a $Mo_{K\alpha}$ fine focus sealed tube (λ = 0.71073 nm) operating at 1.5 kW (50 kV, 30 mA) and filtered with a graphite TRIUMPH monochromator. The structure of **3** was solved using the intrinsic phasing method[3] and subsequent refinements were performed using SHELXL[4] within ShelXle.[5] Crystallographic data for **3** have been deposited with the Cambridge Crystallographic Data Centre (CCDC-1488194) and can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif. Diagrams of complex **3** were generated by ORTEP-3[6] and POV-RAY.[7] Crystal data, data collection parameters and details of structure refinement for complex **3** are listed in **Table S1**.

Optical measurements

UV-Vis spectra were measured using a Cary 100 Bio UV-Visible spectrophotometer with a 6×6 Multicell Block Peltier cooling module, which maintained the sample temperature at 37 °C. Complexes **2** and **3** was first dissolved in DMSO to improve solubility then added to phosphate buffered aqueous solutions, pH 7.4, to give a 200 μM solution. The concentration of chloride in the phosphate buffered solutions was varied as 0, 20 and 139 mM with NaCl. All samples were incubated at 37 °C over of 90 min.

Preparation of $Fe(MGD)_2$

Solutions of $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ and MGD were prepared with PBS degassed with argon and combined immediately before each measurement. These solutions used a 1:5 Fe:MGD ratio to produce a stable spin trap, and had final concentrations of 200 mM Fe and 1000 mM MGD.

EPR sample preparation

Each sample was prepared initially in a 1 mL volume, from which 45 μL was placed in a glass capillary and used for data collection. A sample temperature of 37 °C was obtained using a flow of heated air over the sample inside the EPR resonator. A concentrated DMSO stock solution of complex **3** (250 mM) was used to improve solubility in PBS.

PBS sample: Complex **3** in DMSO (250 mM, 20 μL) and $Fe(MGD)_2$ in PBS (200 mM, 50 μL) were combined and the solution was made up to 1 mL with PBS, to give final concentrations of [**3**] = 5 mM and $[Fe(MGD)_2]$ = 10 mM, with 2% DMSO.

GSH sample: Complex **3** in DMSO (250 mM, 20 μL), $Fe(MGD)_2$ in PBS (200 mM, 50 μL), and GSH in PBS (125 mM, 200 μL) were combined and the solution was made up to 1 mL with PBS, to give final concentrations of [**3**] = 5 mM, $[Fe(MGD)_2]$ = 10 mM, [GSH] = 25 mM.

Serum sample: Complex **3** in DMSO (250 mM, 20 μL), $Fe(MGD)_2$ in PBS (200 mM, 50 μL) were combined and the solution was made up to 1 mL with human serum, to give final concentrations of [**3**] = 5 mM and [$Fe(MGD)_2$] = 10 mM, with 2% DMSO.

EPR measurements

EPR measurements were performed at X-band (9.86 GHz) using a Bruker EMXplus spectrometer with a PremiumX microwave bridge and HS resonator. Experimental parameters: 20 mW microwave power, 6.0 Gauss modulation amplitude, 100 kHz modulation frequency, 5.12 ms time constant, 70 G sweep width, and 10.24 s scan time.

Biological activity testing

A549 adenocarcinomic human alveolar basal epithelial cells were acquired directly from Dr. Marcel Bally's laboratory (BC Cancer Agency Research Center, Vancouver, BC). The A549 cells were cultured at 37 °C under a 5% CO_2 atmosphere in RPMI 1640 media supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Complexes **2** and **3**, and 4-nitrooxymethylpyridine (**1**), were diluted from 10 mM stocks in DMSO to give the desired concentrations in complete cellular media for cytotoxicity testing. To verify that the compounds would remain soluble under assay conditions, each compound was incubated in complete cell media at the maximum testing concentrations for 72 hours at 37 °C and in each case there was no evidence of precipitation.

For *in vitro* cytotoxicity assays, A549 cells were seeded in quadruplet at 2000 cells/well in 384-well plates (Grener Bio-One). Following a 24 h incubation period, 20 μL aliquots of each complex in media were added to each well to give the desired concentration with a DMSO concentration of 1%. Wells containing a media control and a vehicle (DMSO) control were also prepared. After 72 h of treatment with each compound, the cells were stained using 5 μL of a 10 mg/mL stock of Hoescht 33342 nucleic acid stain and 3 μL of a 1 mM stock of ethidium homodimer I per mL of media. These stains generate a total cell count and a dead cell count respectively. After a 20 minute incubation period the plates were then imaged using an IN Cell Analyzer 1000 (GE Healthcare), which is an automated fluorescent microscopy platform that enables high-content screening. Cell counts were determined via the IN Cell Developer Toolbox software. Cells were classified as "dead" if they showed >30% overlap of the two stains. Statistical analyses to determine half maximal inhibitory concentrations (IC_{50}) were performed using GraphPad software.

NMR and IR spectra from ligand 1

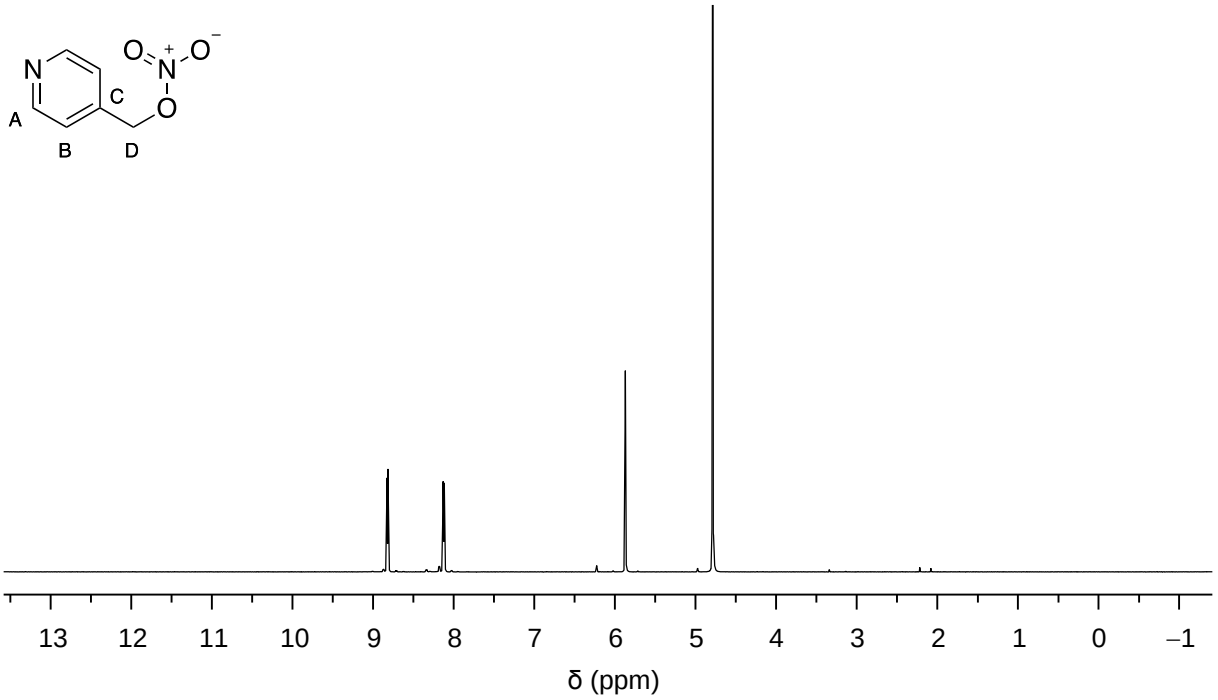

Figure S1: ^{1}H NMR spectrum of compound **1** (500 MHz, D_2O, ppm): δ 8.82 (A, d, J = 6.2 Hz, 2H), 8.12 (B, d, J = 6.1 Hz, 2H), 5.87 (D, s, 2H).

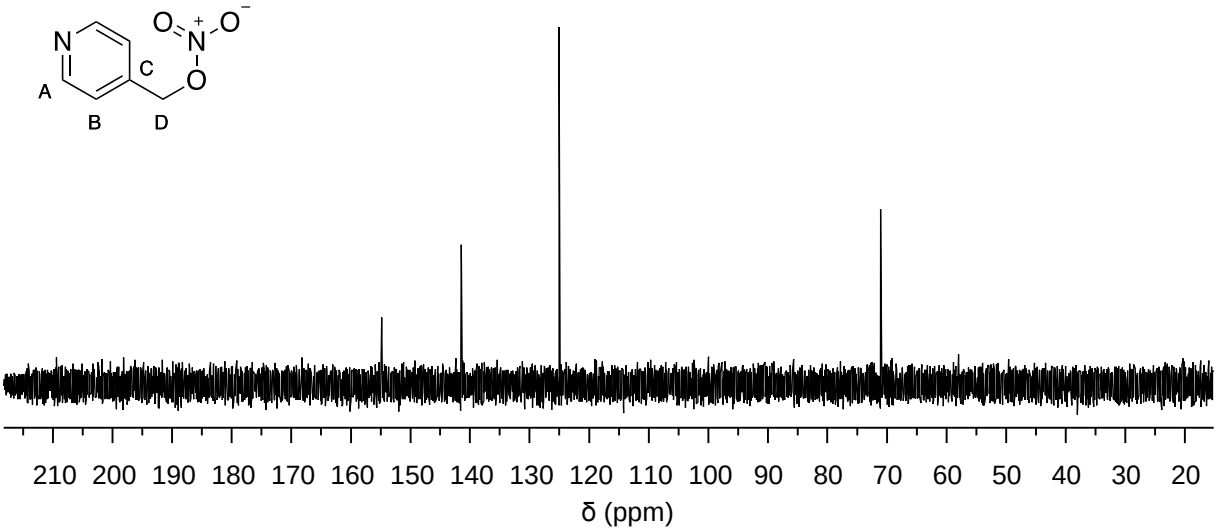

Figure S2: ^{13}C NMR spectrum of compound **1** (126 MHz, D_2O, ppm): δ 154.84 (A), 141.46 (C), 125.04 (B), 71.08 (D).

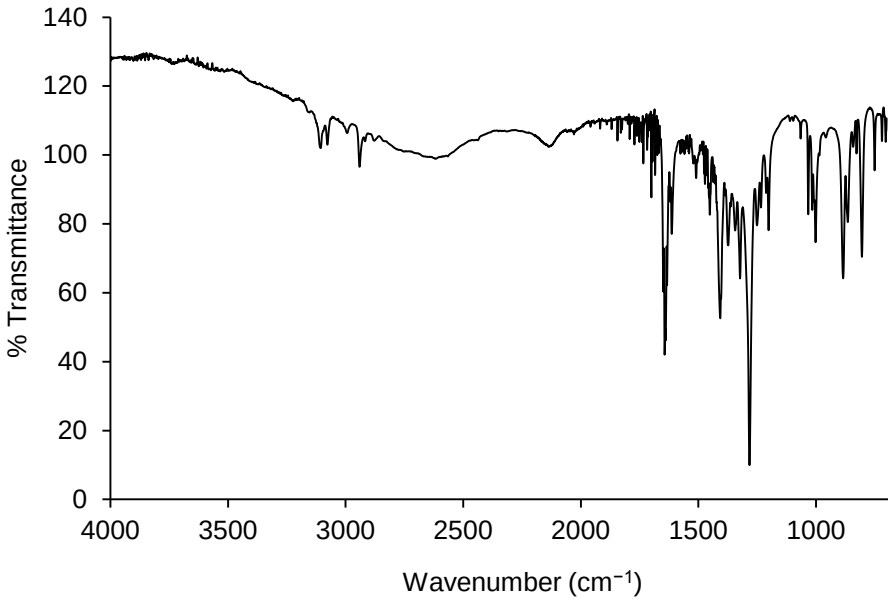

Figure S3: IR spectrum of ligand **1**.

NMR and IR spectra from complex 3

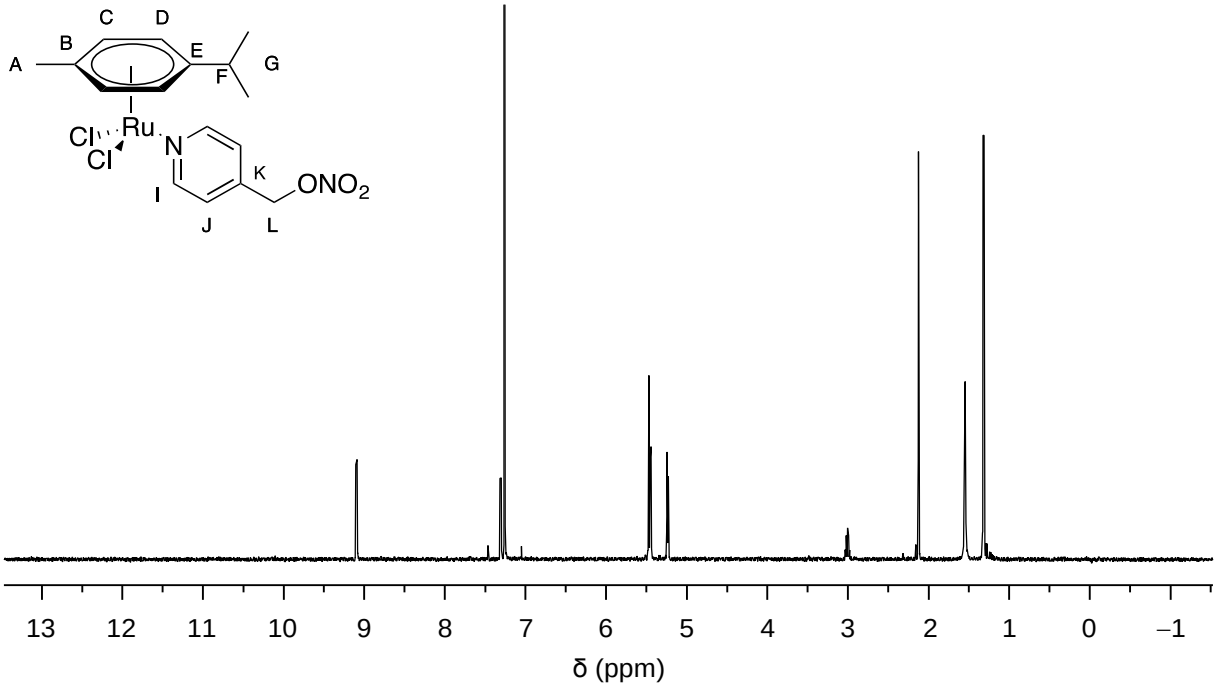

Figure S4: ^{1}H NMR spectrum of complex **3** (500 MHz, $CDCl_3$, ppm): δ 9.10 (I, m, 2H), 7.31 (J, d, J = 6.0 Hz, 2H), 5.47 (L, s, 2H), 5.45 (D, d, J = 5.9 Hz, 2H), 5.23 (C, d, J = 5.9 Hz, 2H), 3.01 (F, m, J = 6.9 Hz, 1H), 2.12 (A, s, 3H), 1.32 (G, d, J = 6.9 Hz, 6H).

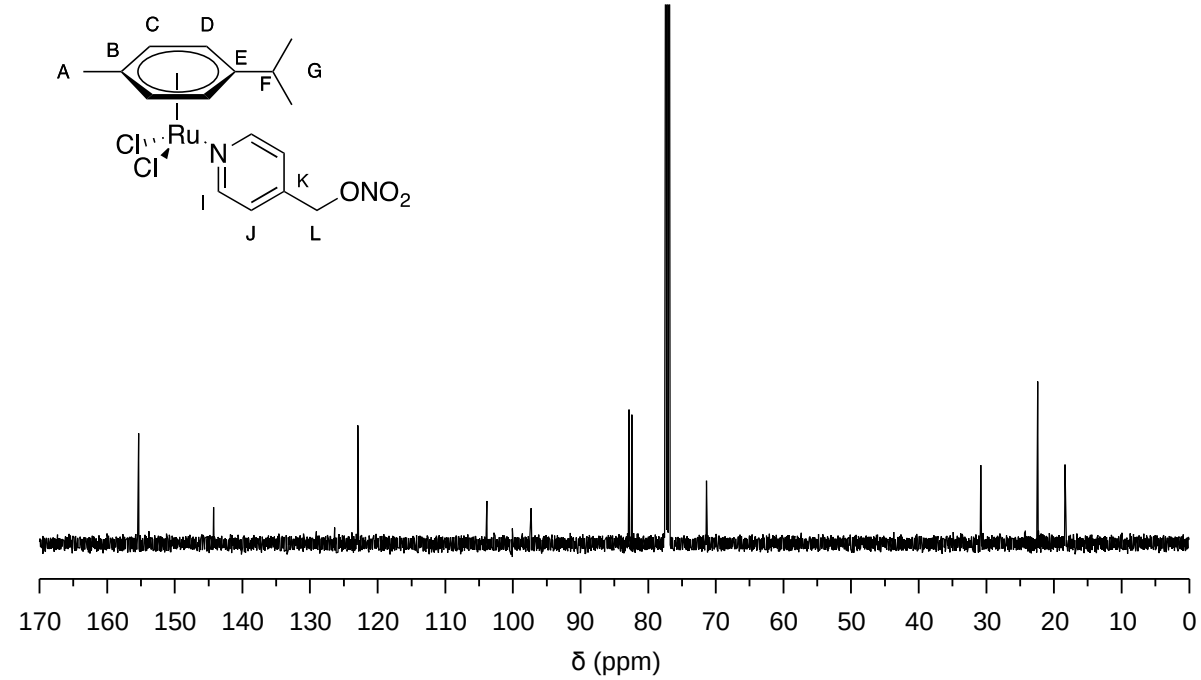

Figure S5: ^{13}C NMR spectrum of complex **3** (126 MHz, $CDCl_3$, ppm): δ 155.35 (I), 144.25 (K), 122.93 (J), 103.86 (E), 97.32 (B), 82.84 (D), 82.41 (C), 71.36 (L), 30.81 (F), 22.40 (G), 18.39 (A).

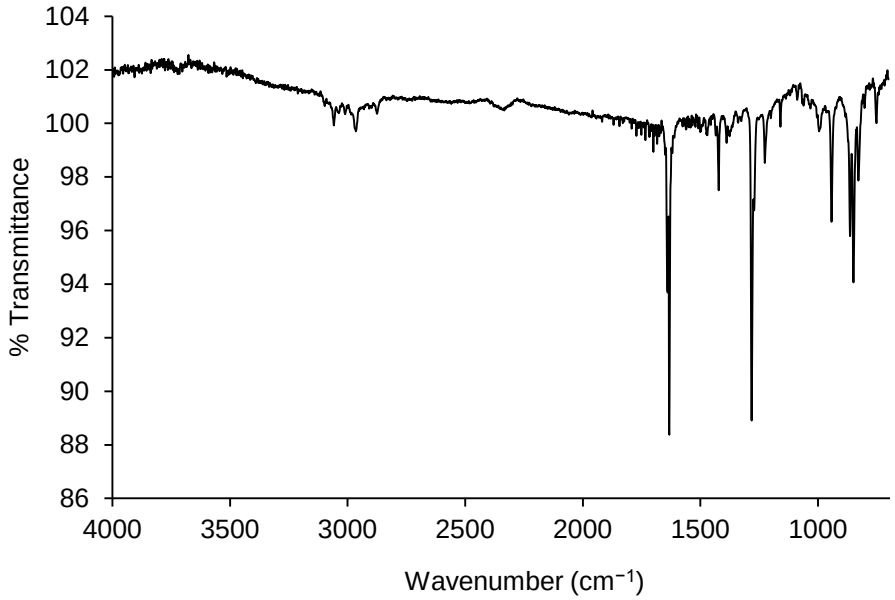

Figure S6: IR spectrum of complex **3**.

Crystal data from complex 3

Table S1. Crystal data and details of data collection and refinement for complex **3**.

Empirical formula	$C_{24}H_{17}Cl_4N_4O_3Ru$
M (g mol^{-1})	579.68
Space group	Triclinic P –1
a (Å)	9.6767(2)
b (Å)	11.0960(3)
c (Å)	12.1079(3)
α (deg)	69.198(1)
β (deg)	84.296(1)
γ (deg)	75.487(1)
V (Å^3)	1176.45(5)
Z	2
ρ_{calc} (g cm^{-1})	1.636
μ (g mm^{-1})	1.254
λ (Å)	0.71073
T (K)	296
Goodness of fit	1.032
R_1	0.0287
wR_2	0.0739

UV-Vis spectra from complex 2

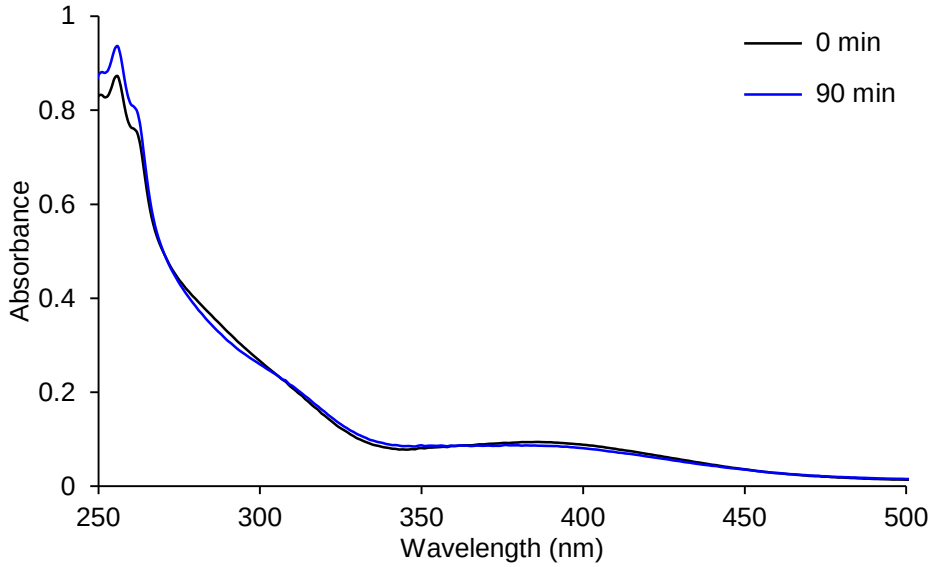

Figure S7: UV-Vis spectra of complex **2** (200 μM) in phosphate buffer, pH 7.4, 1% DMSO, at 37 °C over the course of 90 min.

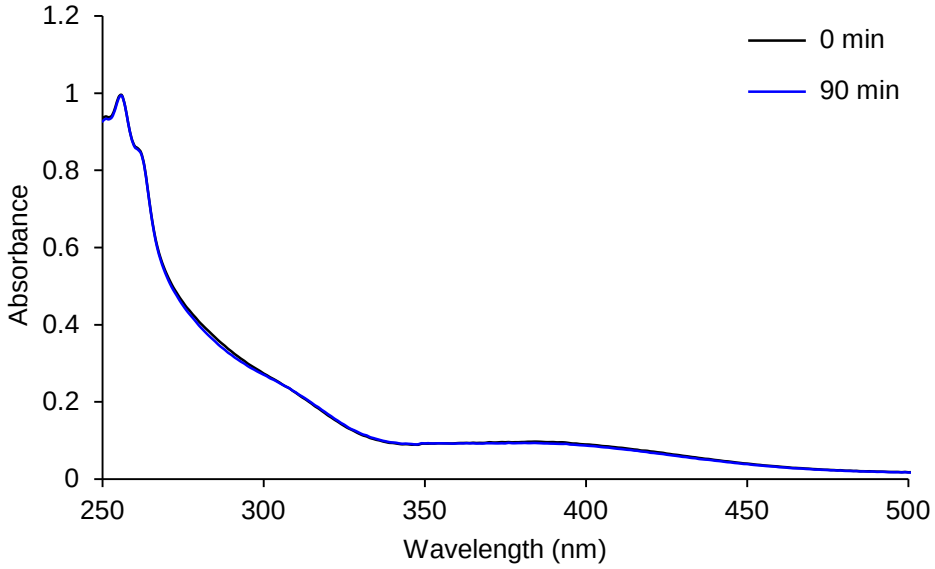

Figure S8: UV-Vis spectra of complex **2** (200 μM) in phosphate-buffered saline, pH 7.4, [Cl^-] = 20 mM, 1% DMSO at 37 °C over the course of 90 min.

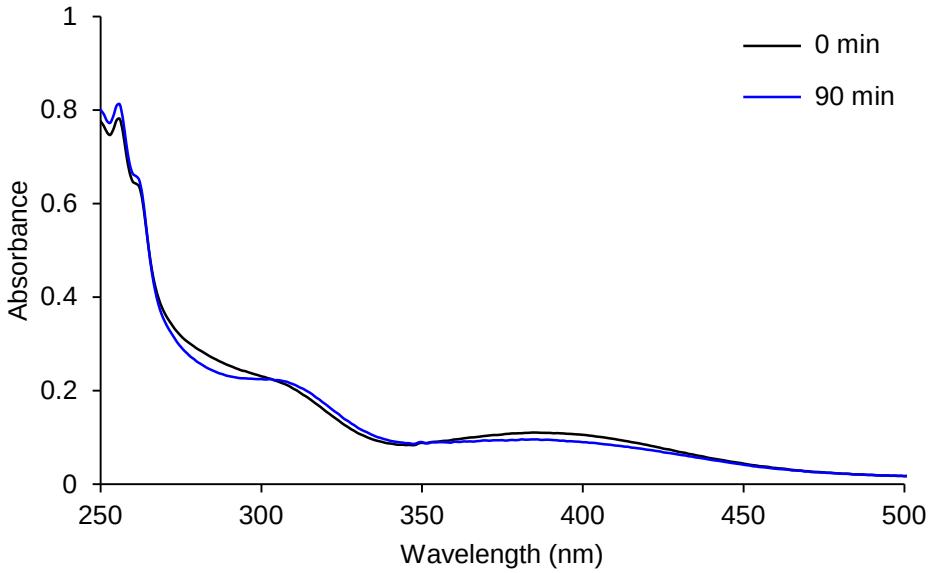

Figure S9: UV-Vis spectra of complex **2** (200 μM) in phosphate-buffered saline, pH 7.4, [Cl^-] = 139 mM, 1% DMSO at 37 °C over the course of 90 min.

UV-Vis spectra from complex 3

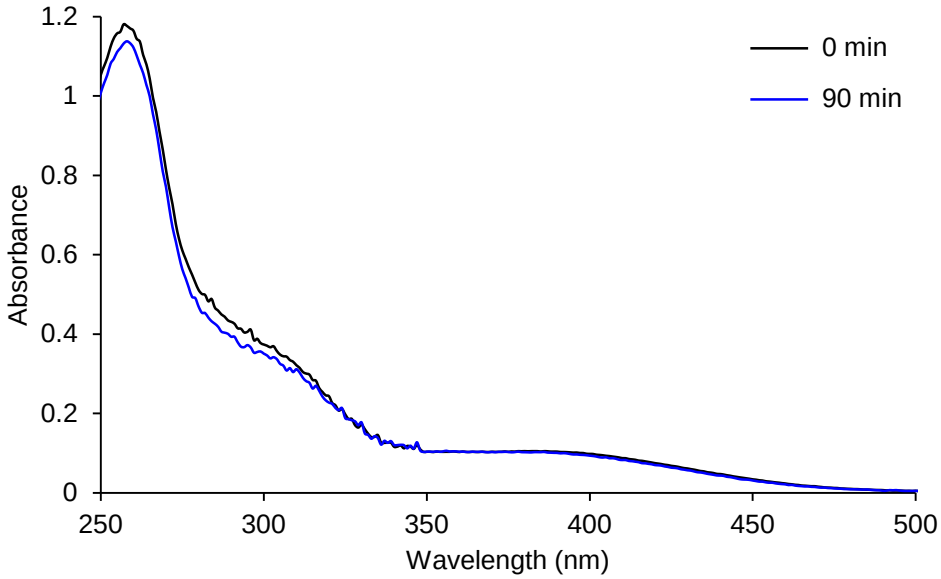

Figure S10: UV-Vis spectra of complex **3** (200 μM) in phosphate buffer, pH 7.4, 1% DMSO, at 37 °C over the course of 90 min.

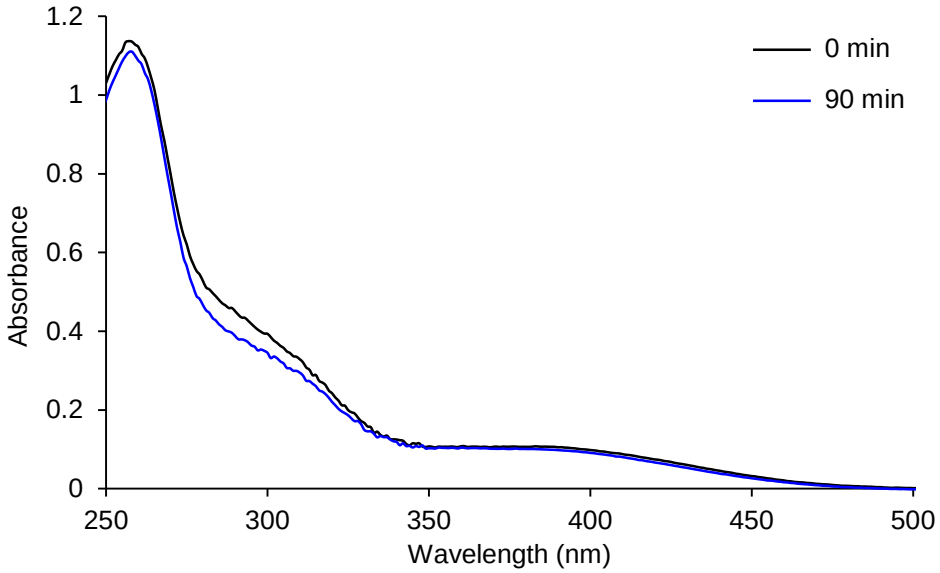

Figure S11: UV-Vis spectra of complex **3** (200 μM) in phosphate-buffered saline, pH 7.4, [Cl^-] = 20 mM, 1% DMSO at 37 °C over the course of 90 min.

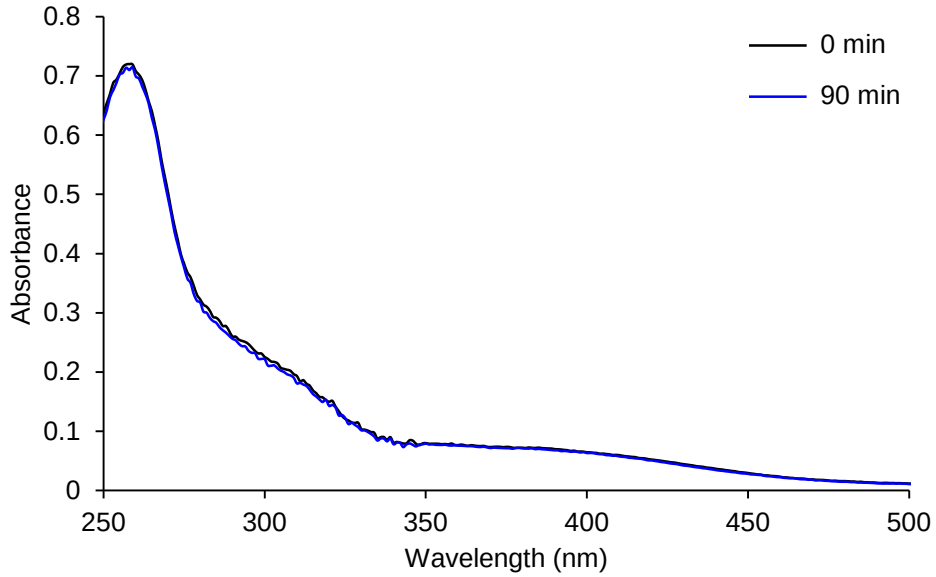

Figure S12: UV-Vis spectra of complex **3** (200 μM) in phosphate-buffered saline, pH 7.4, [Cl^-] = 139 mM, 1% DMSO at 37 °C over the course of 90 min.

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