A luminescent ruthenium(II) complex for light-triggered drug release and live cell imaging

Nora Karaoun and Anna K. Renfrew

1. General Procedures

Materials

Econazole nitrate was obtained from Sigma Aldrich as a 1:1 racemic mixture of the R and S enantiomers and used as received. All other chemicals were obtained from commercial sources and used with further purification.

Instrumentation and Methods

^{1}H NMR spectra were collected at 300 K on a Bruker 300 MHz spectrometer using commercially available deuterated solvents. Isotopic impurities were used as internal reference signals. Mass spectrometry was performed using Electro-Spray Ionisation using a Finnigan LCQ-8 spectrometer. Elemental analyses (C, H, N) were conducted by the Chemical & MicroAnalytical Services Pty Ltd, Campbell Microanalytical Laboratory, at the University of Otago. ICP-MS was conducted at the National Measurements Institute, Pymble, NSW, Australia. UV-visible measurements were performed on a Cary 4E UV-visible spectrometer using a 1 cm x 1 cm quartz cuvette. Scans were run at room temperature from 300 – 700 nm. Fluorescence measurements were performed using a Varian Cary Eclipse fluorescence spectrophotometer, using a 1 cm x 1 cm quartz cuvette. Scans were run at room temperature at 100 nm/min with excitation and emission slit widths of 5 nm or 10 nm. All solutions were prepared immediately prior to analysis. Emission scans were run between 550 and 800 nm

using an excitation wavelength of 490 nm.

2. Synthesis

$[Ru(phen)_2Cl_2]$ and $[Ru(phen)_2(im)_2](PF_6)_2$ [were prepared according to literature procedures.[1,2] $[Ru(phen)_2(im)Cl]PF_6$ was prepared from $[Ru(phen)_2(im)_2](PF_6)_2$ according to a literature procedures.[3] All reactions were carried out under nitrogen using standard Schlenk techniques. The synthesis and purification of the final complexes were performed under low ambient light to avoid photodegradation.

$[Ru(phen)_2(econazole)Cl]PF_6$ (1)

An solution of $[Ru(phen)_2Cl_2]$ (266 mg, 0.5 mmol) in 1:1 water/MeOH (50 mL) was heated at reflux for 30 min in the absence of light. A solution of econazole nitrate (265 mg, 0.6 mmol) in DMF (2 mL) was added and the reaction heated at reflux for a further 8 h. The volume of ethanol was reduced by half and excess NH_4PF_6 added to give a wine red precipitate. The precipitate was collected by filtration and washed with diethyl ether (3 x 20 mL). This reaction gives **1** as the major product and **2** as a minor product. The two complexes were separated on an alumina column (neutral, Brockmann activity 3) with a gradient eluent of diethyl ether:acetonitrile (3:1 to pure acetonitrile). **1** elutes first as a dark red band followed by **2** as an orange band. The fractions were combined, concentrated and precipitated with diethyl ether to give a wine red crystalline solid, which was collected by filtration, washed with diethyl ether (2 x 20 mL) and dried under vacuum. Final yield of **1** = 271 mg (53%) of dark red microcrystals. This product is a 1:1 mixture of $[Ru(phen)_2$ (*r*-Econazole)Cl]PF_6 and $[Ru(phen)_2$ (*s*-Econazole)Cl]PF_6.

^{1}H NMR (300 MHz, Acetonitrile-d_3) 10.26 (2 H, d, *J* 5.4), 8.91 (2 H, t, *J* 5.2), 8.61 (4 H, q, *J* 8.0), 8.34 - 8.24 (4 H, m), 8.21 (2 H, d, *J* 6.0), 8.12 (4 H, d, *J* 8.6), 8.09 - 7.98 (4 H, m), 7.98 - 7.87 (2 H, m), 7.86 - 7.76 (4 H, m), 7.70 (1 H, s), 7.42 (2 H, t), 7.40 - 7.31 (4 H, m), 7.25 (2 H, d, *J* 8.4), 7.14 - 7.06 (4 H, m), 7.03 (2 H, d, *J* 8.9), 6.92 (2 H, d, *J* 8.3), 6.86 (1 H, d, *J* 8.4), 6.78 (1 H, s), 6.75 (1 H, s), 6.64 (1 H, s), 6.52 (1 H, s), 4.92 (1 H, t, *J* 5.2), 4.82 (1 H, t, *J* 5.2), 4.36 - 3.99 (8 H, m). ESI-MS$^+$: m/z = 878.53 ($[Ru(phen)_2(Ec)Cl]$)$^+$. Elemental analysis for $[Ru(phen)_2(Ec)Cl]PF_6 \bullet (H_2O)$ ($C_{43}H_{32}Cl_4F_6N_6O_2PRu$). Calculated: C, 49.07; H, 3.06; N, 7.98. Found: C, 49.00; H, 3.08; N, 8.04

$[Ru(phen)_2(econazole)_2](PF_6)_2$ (2)

An ethanol solution (50 mL) of $[Ru(phen)_2Cl_2]$ (266 mg, 0.5 mmol) and $AgBF_4$ (213 mg, 1.1 mmol) was heated at reflux for 15 min in the absence of light. A solution of econazole nitrate (663.9 mg, 1.5 mmol) in DMF (2 mL) was added and the reaction heated at reflux for a further 8 h. The red solution was filtered to remove AgCl then the volume of ethanol was reduced by half and an equivalent volume of aqueous added NH_4PF_6 to give an orange/red precipitate. The precipitate was collected by filtration and washed with diethyl ether (3 x 20 mL). This reaction gives **2** as the major product and **1** as a minor product. The product was purified on an alumina column as described above for complex **1**. The second (orange) band was collected and the fractions were combined, concentrated and precipitated with diethyl ether to give a brick red crystalline solid. This was collected by filtration, washed with diethyl ether (2 x 20 mL) and dried under vacuum. Final yield of **2** = 335 mg (44%) of

orange/red microcrystals. This product was isolated as mixture of diastereomers at a ratio of 4:5 (see supporting Figure S1).
^{1}H NMR (300 MHz, Acetonitrile-d_3) 9.28 (2 H, d, *J* 5.4), 9.24 (2 H, d, *J* 5.3), 8.74 - 8.64 (4 H, m), 8.39 - 8.29 (4 H, m), 8.20 (2 H, d, *J* 2.7), 8.17 (2 H, d, *J* 2.7), 8.14 – 7.95 (12 H, m), 7.48 - 7.37 (6 H, m), 7.36 (2 H, d, *J* 2.2), 7.23 – 7.21 (4 H), 7.14 (2 H, s), 7.12 (1 H, s), 7.04 - 6.93 (6 H, m), 6.92 (1 H, s), 6.89 (6 H, s), 6.87 (6 H, d, *J* 2.9), 6.84 (2 H, d, *J* 6.0), 6.76 - 6.80 (4 H), 6.71 (1 H, d, *J* 3.3), 6.66 (2 H, s), 6.55 (1 H, d, *J* 8.4), 6.53 (1 H, s), 6.46 (1 H, s), 4.80 (2 H, dt, *J* 9.1, 4.8), 4.76 - 4.67 (2 H, m), 4.27 - 4.20 (2 H, m), 4.19 (5 H, s), 4.17 (2 H, s), 4.16 - 4.08 (5 H, m), 4.08 - 3.95 (3 H, m).
ESI-MS$^+$: m/z = 611.80 $([Ru(phen)_2Ec_2])^{2+}$. Elemental analysis for $[Ru(phen)_2(Ec)_2]PF_6 \bullet (CH_3CN,(C_2H_5)_2O)$ ($C_{66}H_{59}Cl_6F_{12}N_9O_3P_2Ru$). Calculated: C, 48.63; H, 3.65; N, 7.73. Found: C, 48.30; H, 3.60; N, 7.76

Counter Ion Exchange
All complexes were converted to chloride salts for spectroscopic measurements and biological testing according to a reported procedure.[4] The complex as a PF_6 salt was dissolved in the minimum volume of acetone. A saturated acetone solution of tertbutylammonium chloride was added dropwise, resulting in precipitation of the complex as a chloride salt. The precipitate was collected by filtration, washed with acetone, and dried.

Aquation of complex 1
$[Ru(phen)_2(econazole)H_2O]Cl_2$ was prepared by refluxing a solution of **1** in 1:1 methanol/water (50 mg /50 mL) for 1h. The solvent was removed at reduced pressure until precipitation of $[Ru(phen)_2(econazole)H_2O]Cl_2$ as a brick red powder. The precipitate was collected by suction filtration and washed with acetone and dried. Exchange of the chloride ion with was confirmed by ESI-mass spectrometry and UV-vis absorbance spectroscopy.

3. Spectroscopic studies

Luminescence quantum yield
Luminescence quantum yields were determined according to the following equation where Φ_s and Φ_r are the quantum yields of the sample and reference respectively, I is the integrated luminescence emission on excitation at 488 nm and A is the absorbance at 488 nm.

$$\Phi_s = \Phi_r (I_s/I_r) (A_r/A_s)$$

$[Ru(bpy)_3](Cl)_2$ in water was used as a reference with a reported quantum yield of 0.042.[5] Solutions of all complexes were prepared in aerated water (1% DMSO) at a concentration of 50 μM.

Stability and photoreactivity studies
Solutions of the ruthenium complexes in water (1% DMSO) were prepared in a quartz cuvette to give a final concentration of 50 – 70 μM. The cuvette was placed in an ice bath inside a box protected from ambient light and irradiated with a multi LED lamp (120 x 3 W LED diodes, λ_{LED} = 520 ± 20 nm) with a fluence rate of 53 J cm^{-2} h^{-1}. UV-visible absorbance and emission spectra were recorded at regular intervals and ESI mass spectra of the initial and final solutions were collected.

The quantum yield for the photoinduced ligand exchange of the first econazole ligand in **2** was determined according to a reported procedure by monitoring the decrease in absorbance at 486 nm as a function of irradiation time.[6] Ferrioxalate actinometry was used to determine the photon flux of the LED light source.[7] The quantum yield of photolysis was determined by plotting the decrease in the number of moles of complex per unit time (determined from the UV-visible absorbance maxima by c = A/εl) against the number of moles of photons (Fig. S4d) during the initial 20% of the photoreaction. The slope of the plot gives the quantum yield.

4. Biological studies

Cell lines

DLD-1 human colon carcinoma, PC-3 and LNCaP human prostate carcinoma, and MCF-7 human breast carcinoma cells were purchased from ATCC and used within 2 months of resuscitation. DLD-1 cells were maintained in Advanced DMEM (Invitrogen) and supplemented with 2% FBS and 2mM glutamine in a humidified environment at 37 ^{o}C and 5% CO_2. PC-3 cells were maintained in Advanced RPMI (Invitrogen) and supplemented with 2% FBS and 2mM glutamine in a humidified environment at 37 ^{o}C and 5% CO_2. LNCap cells were maintained in Advanced RPMI (Invitrogen) and supplemented with 4% FBS and 2mM glutamine in a humidified environment at 37 ^{o}C and 5% CO_2. MCF-7 cells were maintained in EMEM (Invitrogen) and supplemented with 4% FBS and 2mM glutamine in a humidified environment at 37 ^{o}C and 5% CO_2.

Photocytotoxicity assay

Cytotoxicity was determined using the MTT assay. 1 x10^5 (DLD-1 and PC-3) cells or 2 x10^5 (MCF-7 and LNCaP) cells per well were plated on to 96-well plates and allowed to adhere overnight. Freshly prepared media/DMSO (90:10) solutions of the complexes and econazole were added to triplicate wells at concentrations spanning a 4-log range (final DMSO concentrations <0.5%) and incubated in the dark for 24 h. The media was removed and replaced with phenol red free DMEM (Invitrogen) (100 μL per well) and the cells irradiated for 15 minutes with a multi LED lamp (120 x 3 W LED diodes, λ_{LED} = 520 ± 20 nm), or incubated in dark for the same time period. The phenol red free DMEM was removed and replaced with advanced DMEM, advanced RPMI or EMEM, and the cells incubated in the dark for a further 20 h, following which, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (1.0 mM) was added to each well and the cells incubated for 4 h. The culture medium was removed and the resulting purple precipitate dissolved in DMSO (100 μL). The absorbance measured at 600 nm using a Victor^{3}V microplate reader (Perkin Elmer). At least three independent experiments were performed for each compound with triplicate readings in each experiment. IC_{50} values were determined as the drug concentrations required to reduce the absorbance to 50% of that of the untreated control wells. The viability of untreated control cells was determined with and without light irradiation to establish the effect of the green light. The viability of DLD-1 and PC-3 cells was not affected by the light treatment alone but MCF-7 and LNCaP cells showed up to 10% decrease in viability under the same conditions. To remove possible effects of the light treatment, all IC_{50} values were determined relative to the viability of the appropriate light or dark control cells. IC_{50} values were

calculated by fitting the data to a sigmoidal dose response curve using Prism 6 Software.

Preparation of monolayer cell samples for imaging

5 x10^3 DLD-1 cells were plated on to 2 mL Matek dishes and allowed to adhere overnight. The cells were treated with advanced DMEM media/DMSO (90:10) solutions of **2** to give a final concentration of 20 uM (0.5% DMSO). After 4 hrs, the media was removed, cells were rinsed 3 times with PBS. For live cell imaging, fresh phenol red free DMEM was added to the cells, and the samples were imaged immediately. A heated stage (Linkam Scientific) was used to maintain the temperature at 37 °C during imaging. SYTO® 21 nucleic acid stain and Mitotracker® green were obtained from Invitrogen and used according to the manufacturers' instructions. For fixed samples, 1 mL of 4% paraformaldehyde in HEPES buffer was added to the cells after the final rinse with PBS. The samples were incubated for 20 minutes at room temperature then rinsed 3 times with PBS.

Imaging

Confocal images were aquired using an Olympus FluoView FV1000 inverted light fluorescence and confocal microscope and UPLSAPO 60X water-immersion objective lens (NA = 1.20). A SapphireTM 488-20 optically pumped semiconductor laser system was used to excite the samples. The emission ranges were 600 -700 nm (10% laser 630 power, greyfield 130) for complex **2** and 500- 550 nm (10% laser 500 power) for SYTO® 21 nucleic acid stain and Mitotracker® green. At least 3 images were taken per slide and repeated on at least 3 different occasions.

Determination of fluorescence intensities after light treatment

Cells were treated with **2** (20 uM) for 4 h as described above. The cells were washed and fresh phenol red free DMEM added, following which, samples were irradiated for 0, 2, 7, or 15 minutes a multi LED lamp (120 x 3 W LED diodes, λ_{LED} = 520 ± 20 nm), corresponding to doses of 0, 1.7, 6.2 and 13.3 J cm^{-2} respectively. The media was removed and replaced with fresh phenol red free DMEM and the cells imaged directly as described above. 3 independent experiments were performed for each treatment. Quantification of the fluorescence intensity was carried out using ImageJ by drawing a 20 μm^2 square over a representative portion of the image and measuring the integrated fluorescence intensity. Measurements were taken from at least 5 different images in each treatment group

Measurement of intracellular ROS production

1 x10^4 MCF-7 cells were plated on to 2 mL Matek dishes and allowed to adhere overnight, then incubated with 20 μM of **1**, **2** or Econazole nitrate for 4 h. For the dark samples, the media was replaced with phenol red free DMEM and the cells were incubated with 20 μM H_2DCFDA (Sigma-Aldrich) for 30 minutes at 37 °C. The light samples were irradiated for 15 minutes as described above, following which, the media was replaced with fresh phenol red free DMEM and the cells were incubated with 20 μM H_2DCFDA for 30 minutes at 37 °C. The media was removed and replaced with fresh phenol red free DMEM and the cells imaged directly as described above with excitation set at 488 nm and emission at 530 nm. Three independent experiments were performed for each treatment. Quantification of the fluorescence intensity was carried out using ImageJ by drawing a 10 μm^2 square over a representative portion of the image and measuring the integrated fluorescence

intensity. Measurements were taken from at least 6 different images in each treatment group.

5. Supporting Figures

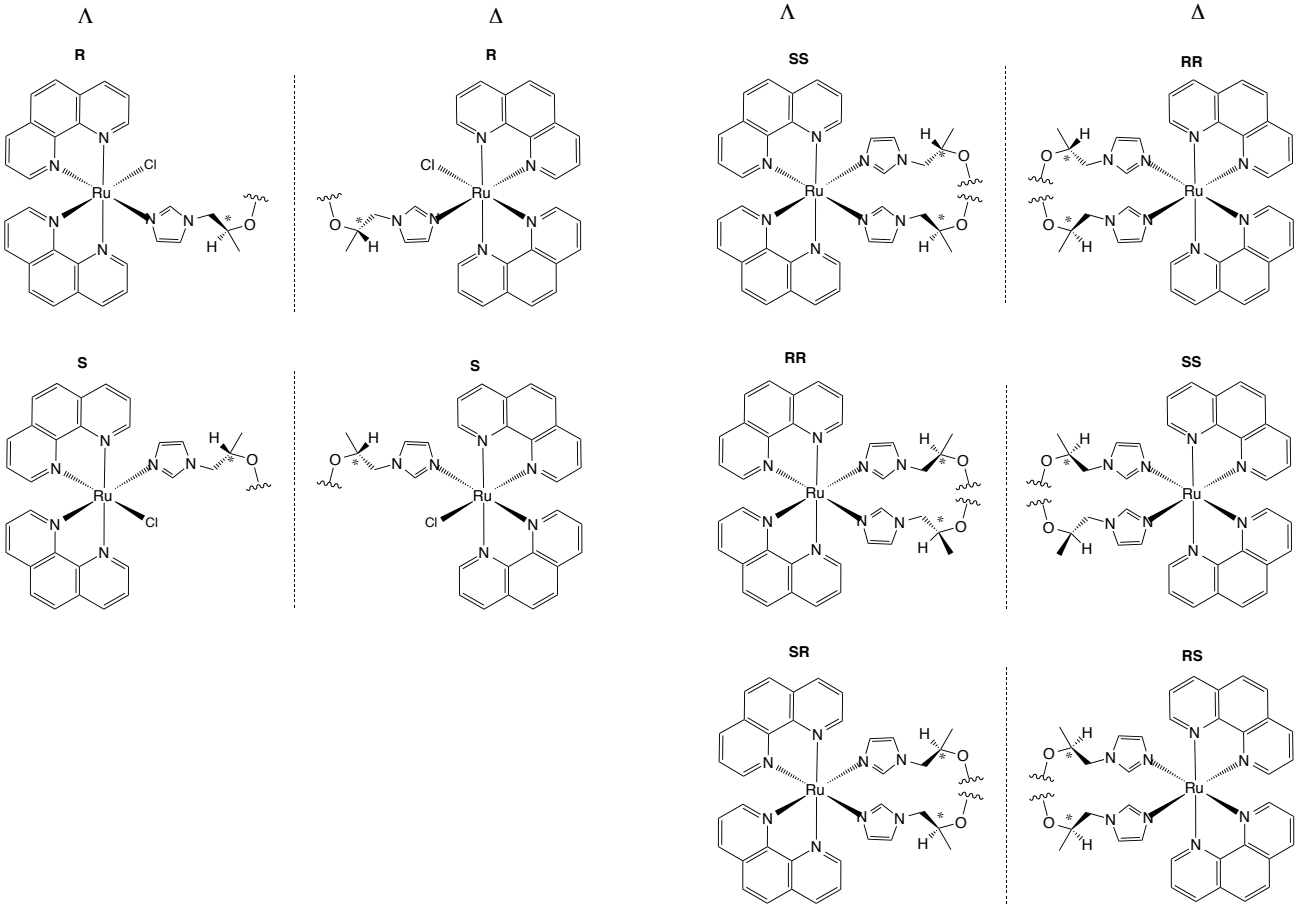

Figure S1: Possible isomers of complex **1** (left) and complex **2** (right).

Econazole has a chiral centre and is administered in the clinic as a racemic mixture of the R and S enantiomers. The combination of a racemic mixture of econazole nitrate with a racemic mixture of the Λ and Δ enantiomers of $[Ru(phen)_2Cl_2]$, yields two pairs of enantiomers for complex **1** (Λ, S and (Δ, S), (Λ, R) and (Δ, R), and three possible pairs of enantiomers for complex **2**: two diasteromers, and a stereoisomer (Λ, S, S) and (Δ, R, R), (Λ, S, S) and (Δ, R, R), (Λ, S, R) and (Δ, S, R) (Fig. S1, ESI†). While the chirality of ruthenium polypyridyl complexes is known to influence interactions with DNA, it was not expected to significantly contribute to the photolabile nature of the ruthenium-econazole complexes and experiments were conducted using the mixture of isomers. Furthermore, by retaining the mixture of isomers, photoactivation results in release of a racemic mixture of econazole, which is consistent with its clinical use.

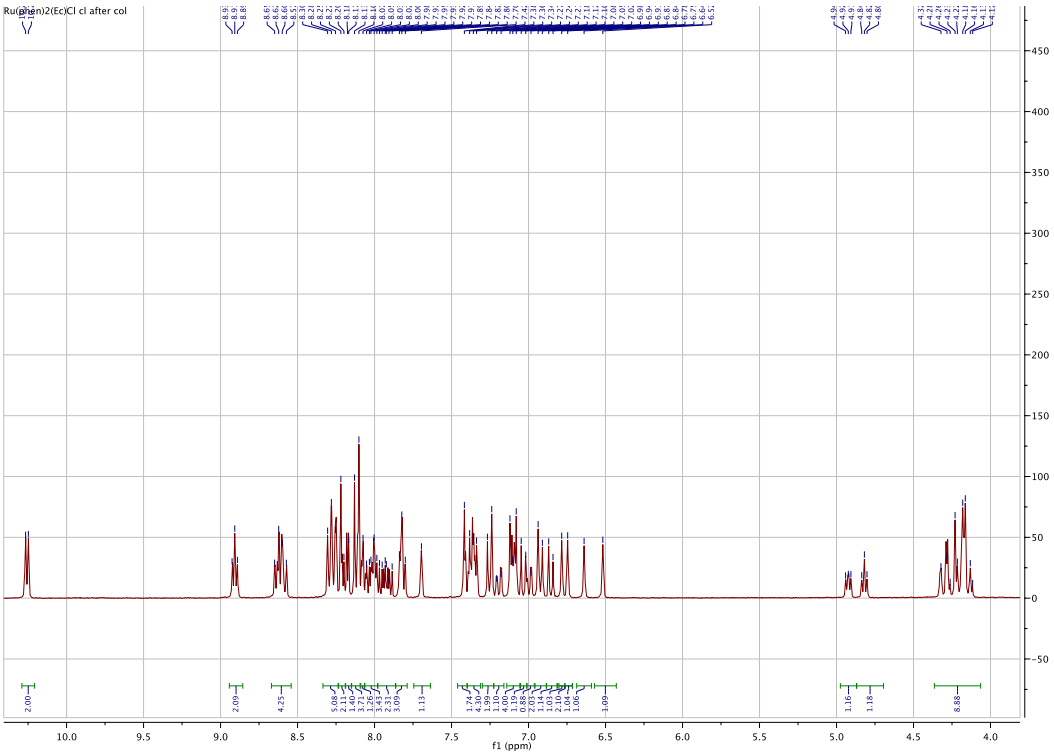

Figure S2a: Aromatic regions demonstrating diastereotopic peaks in the ^{1}H NMR spectra of complex **1**.

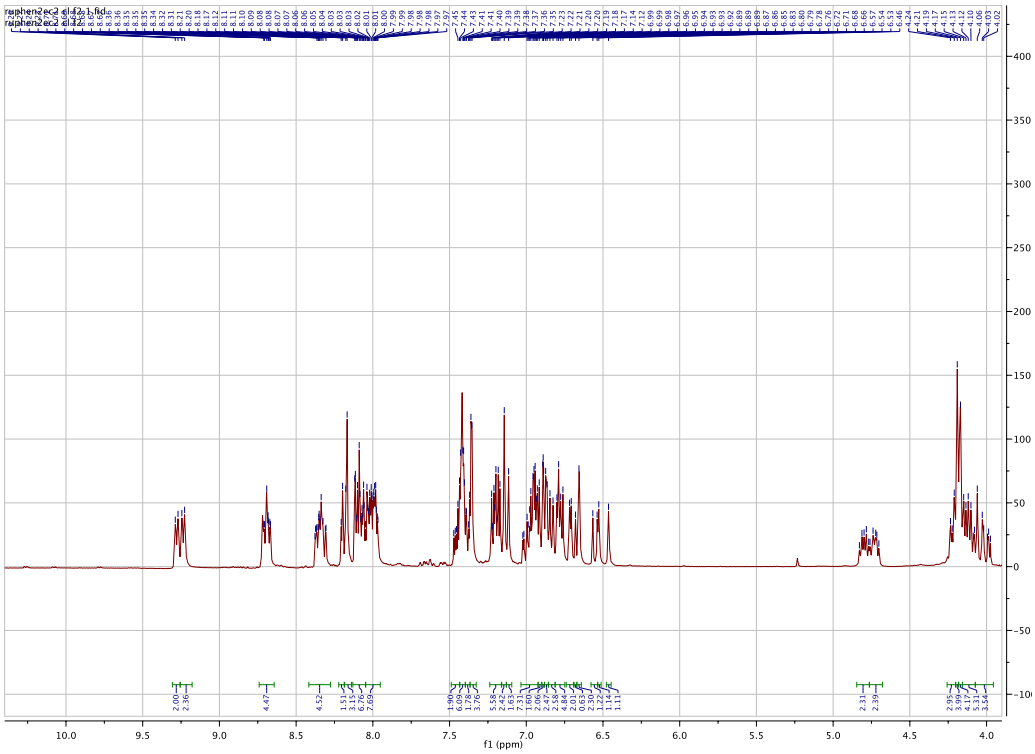

Figure S2b: Aromatic regions demonstrating diastereotopic peaks in the ^{1}H NMR spectra of complex **2**.

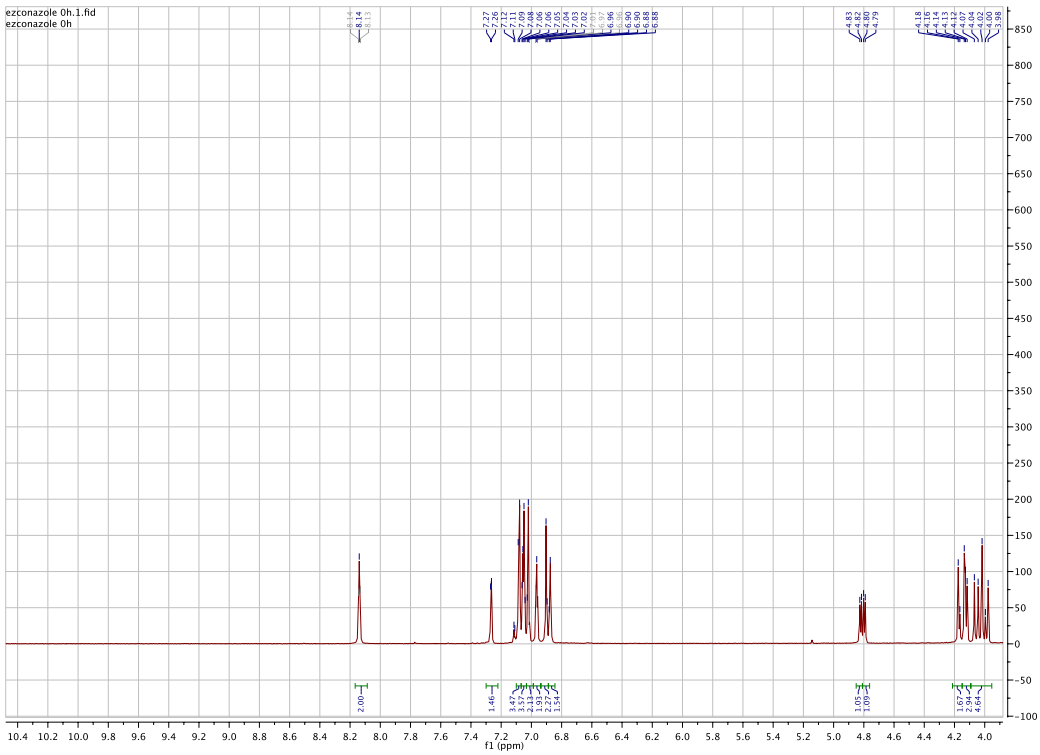

Figure S2c: ^{1}H NMR spectra of the econazole nitrate starting material as a racemic mixture

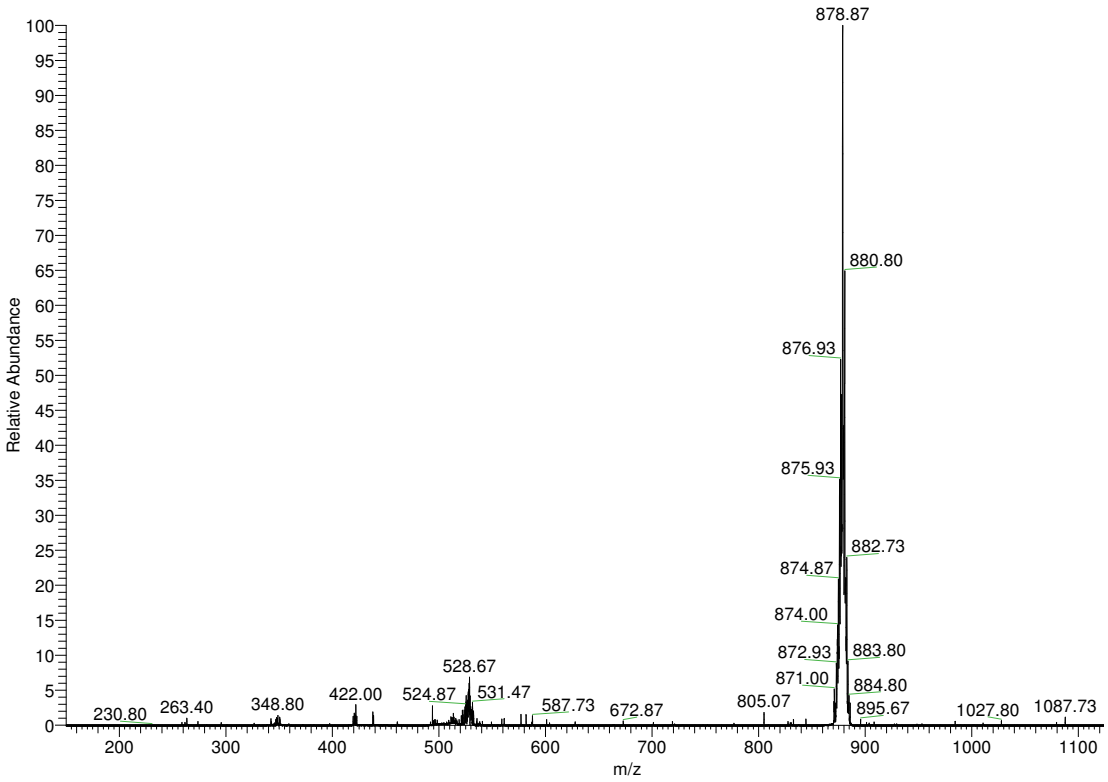

Figure S3a: ESI- mass spectrum of complex **1**.

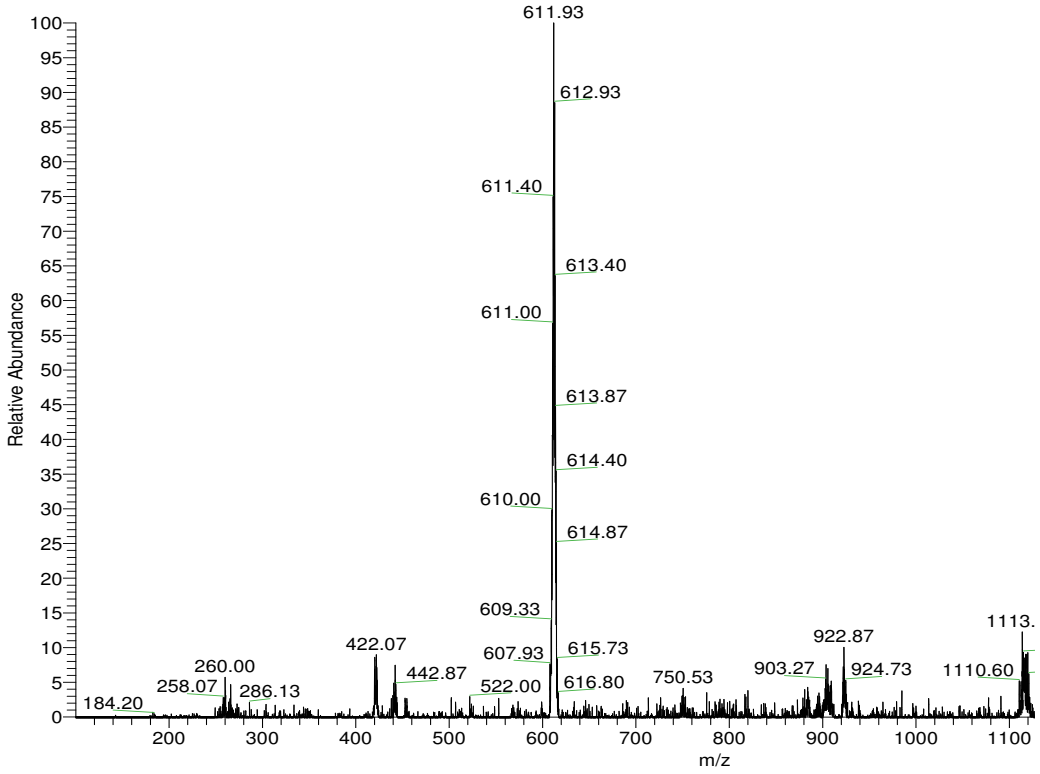

Figure S3b: ESI- mass spectrum of complex **2**

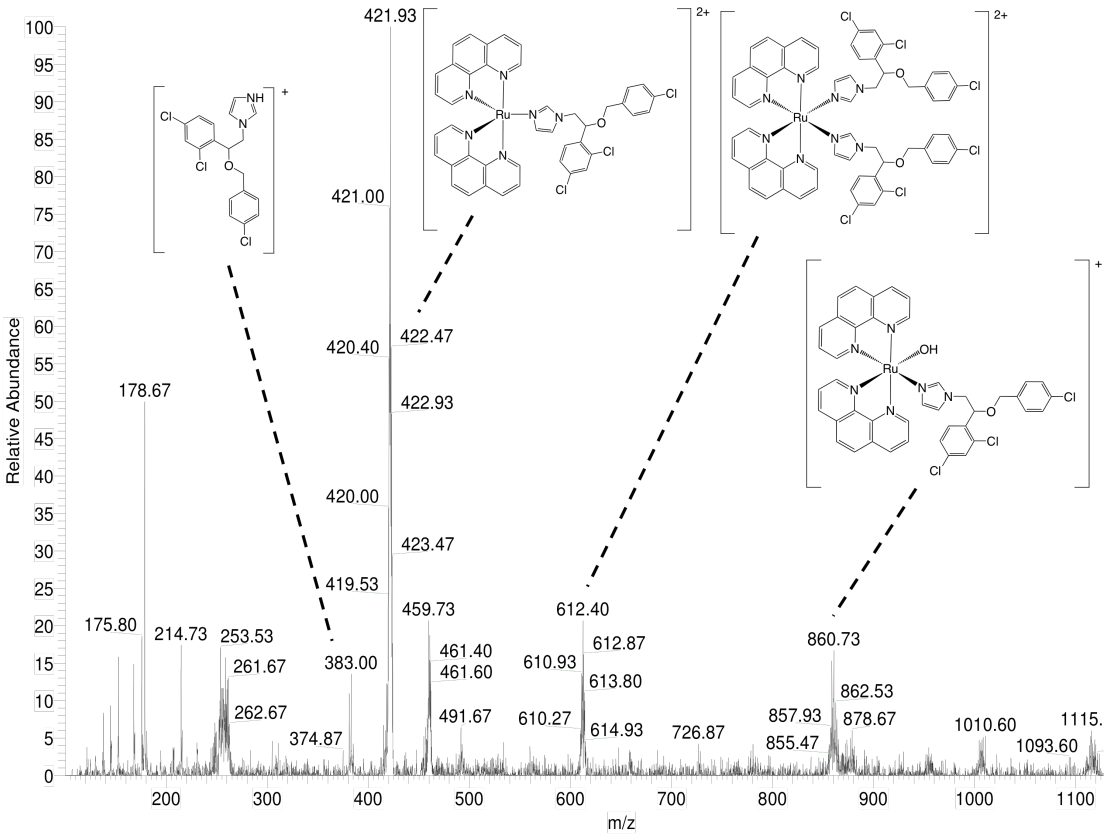

Figure S3c: ESI- mass spectrum of complex **2** after 1 h irradiation

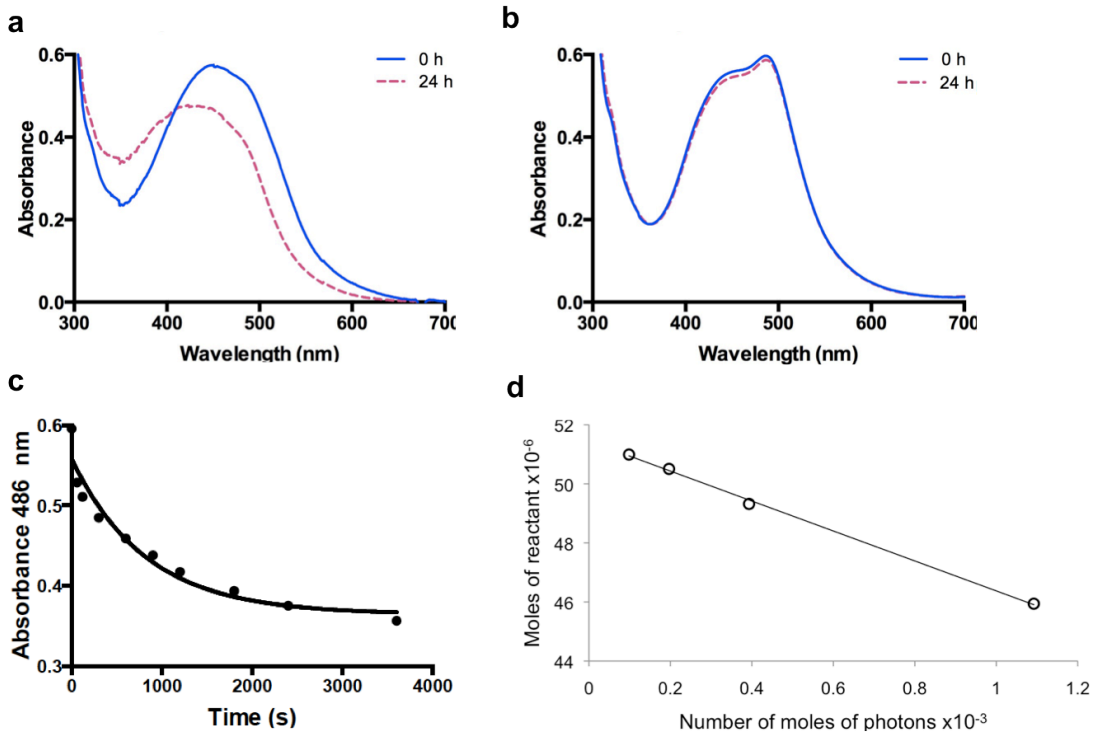

Figure S4: UV-visible absorbance spectra and quantum yield calculation for **1** and **2**. a) UV-visible absorbance spectra of a solution of complex **1** (50 µM in water, 2% DMSO) after 0 and 24h in the dark. b) UV-visible absorbance spectra of a solution of complex **2** (50 µM in water, 2% DMSO) after 0 and 24h in the dark. c) Decrease in absorbance maxima of a solution of complex **2** (50 µM in water, 2% DMSO) with increasing irradiation time. d) Linear fit of the number of moles of **2** against the number of moles of photons. The slope is the quantum yield.

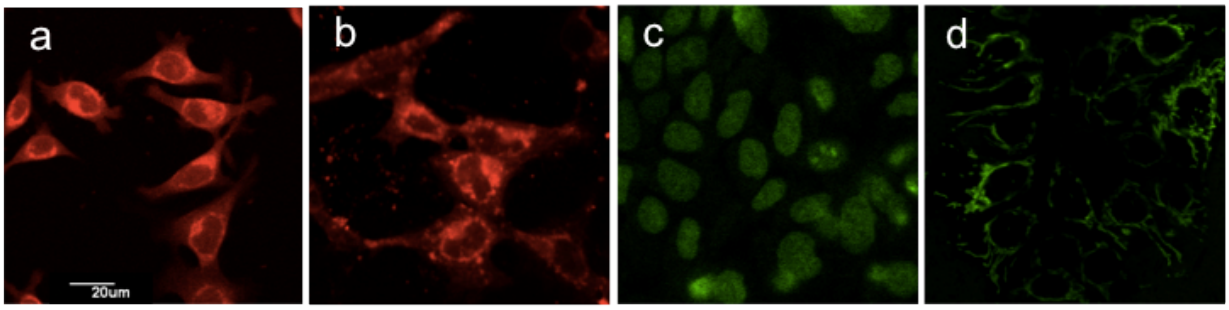

Figure S5: Confocal fluorescence microscopy images of DLD-1 cells a) Cells incubated with **2** (20 µM for 4 h) then fixed with paraformaldehyde. b) Live cells incubated with **2** (20 µM for 4 h). c) Live cells incubated with SYTO® 21 nucleic acid stain d) live cells incubated with Mitotacker green®. Scale bar = 20 µm.

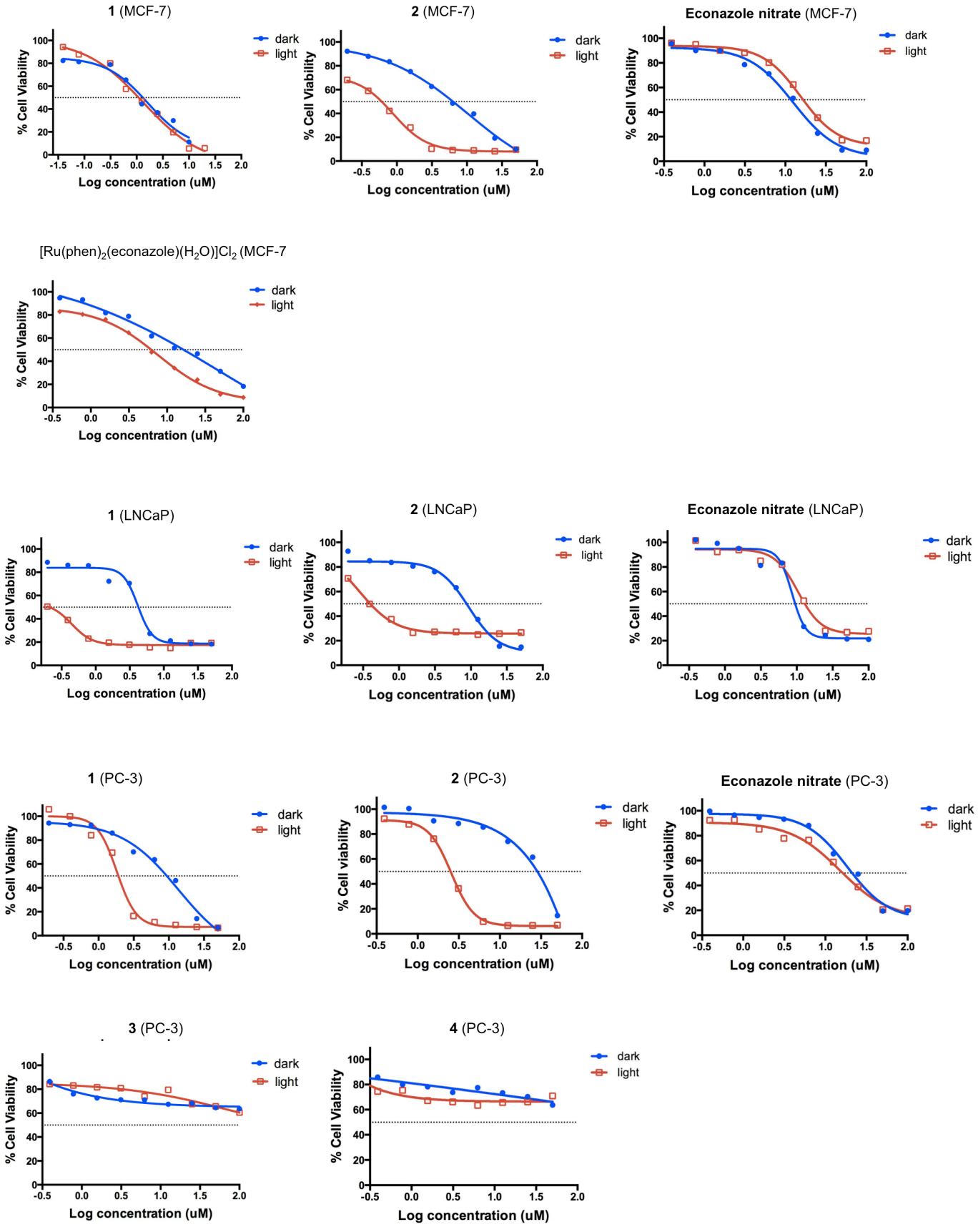

Figure S6a: Sigmoidal fits of cell viability plots in MCF-7, LNCaP and PC-3 cells.

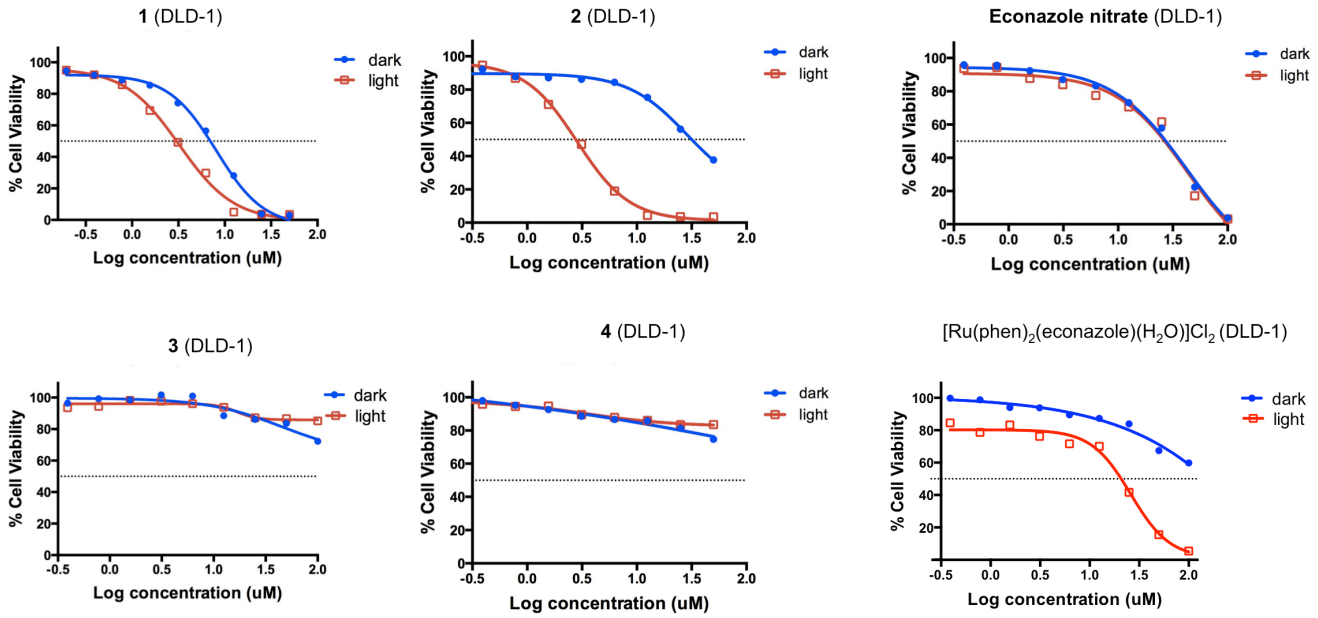

Figure S6b: Sigmoidal fits of cell viability plots in DLD-1 cells.

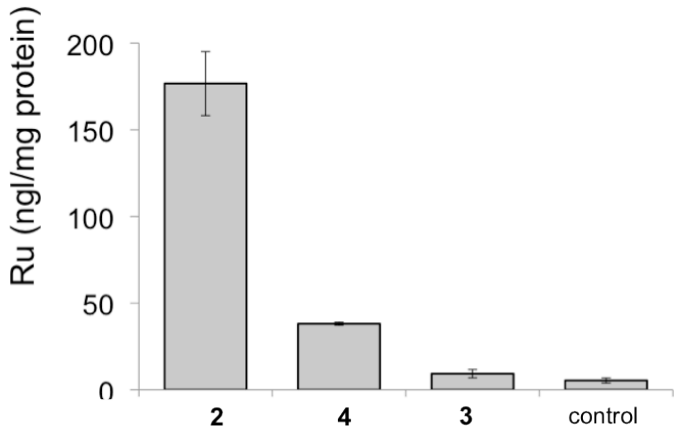

Figure S7: Intracellular ruthenium concentrations in DLD-1 cells

Table S1: Photophysical and photochemical properties of **1** and **2** in water.

Complex	λ_{abs} (nm)	ε ($M^{-1} \bullet cm^{-1}$)	λ_{em} (nm)	$\Phi_{Luminescence.}$ [c]	$\Phi_{Photolysis}$ [c]
1	454	8981	715[a]	<0.001	<0.001
2	486	9570	636[b]	0.067	0.005

a) λ_{ex} = 450 nm; b) λ_{ex} = 488 nm; a) λ_{irr} = 520 nm

Table S2: IC_{50} values (μM) and photoselectivity indexes (PI) in tumour cells. PI = dark IC_{50} value / light IC_{50} value

Compound	**MCF-7**			**PC-3**			**DLD-1**		
	Dark	Light	**PI**	Dark	Light	**PI**	Dark	Light	**PI**
3	n.d	n.d	**-**	>100	>100	**-**	>100	>100	**-**
4	n.d	n.d	**-**	>100	>100	**-**	>100	>100	**-**
$[Ru(phen)(ec)H_2O]Cl_2$	45.8±6.3	7.56±1.7	**6**	n.d	n.d	**-**	>100	25.9±3.6	**≥3.9**

6. References

1. M. R. Norris, J. J. Concepcion, C. R. K. Glasson, Z. Fang, A. M. Lapides, D. L. Ashford, J. L. Templeton and T. J. Meyer, *Inorg. Chem.*, 2013, **52**, 12492-12501.
2. C. R. Cardoso, M. V. S. Lima, J. Cheleski, E. J. Peterson, T. Venancio, N. P. Farrell and R. M. Carlos, *J. Med. Chem.*, 2014, **57**, 4906-4915.
3. X. Hua and A. G. Lappin, *Inorg. Chem.*, 1995, **34**, 992-994.
4. E. Wachter, D. K. Heidary, B. S. Howerton, S. Parkin and E. C. Glazer, *Chem. Commun.*, 2012, **48**, 9649-9651.
5. H. Ishida, S. Tobita, Y. Hasegawa, R. Katoh and K. Nozaki, *Coordin. Chem. Rev.*, 2010, **254**, 2449-2458.
6. E. K. Barry C. Pemberton, Steffen Jockusch, D. K. Srivastava, J. Sivagurua, *Can. J. Chem.,* 2011, **89**, 310-316.
7. A. C. M. Montalti, L. Prodi, M. T.Gandolfi, *Handbook of Photochemistry, Third Edition*, CRC Press, Florida, 2006.