Supporting Information:

1- and 2-Photon Phototherapeutic Effects of Ru(II) Polypyridine Complexes in the Hypoxic Centre of Large Multicellular Tumour Spheroids and Tumour-Bearing Mice

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EXPERIMENTAL SECTION

Materials

All chemicals were obtained from commercial sources and used without further purification. Solvents were dried over molecular sieves if necessary. The ligands \((E,E')-4,4'\)-Bis\(p\)-(N,N-dimethylamino)styryl\)-2,2’-bipyridine and \((E,E')-4,4'\)-Bis\(p\)-methoxystyryl\)-2,2’-bipyridine were synthesised as previously reported\(^{[1]}\). The Ru(II) precursors and Ru(phen)\(_2\)Cl\(_2\) and Ru(bphen)\(_2\)Cl\(_2\) were synthesised as previously reported using the respective ligands.\(^{[2]}\)

Dulbecco’s Modified Eagles Medium (DMEM), Dulbecco’s Modified Eagles Medium supplemented with nutrient mixture F-12 (DMEM/F-12), Gibco Minimum Essential Media (MEM), Fetal Bovine Serum (FBS), Gibco Penicillin-Streptomycin-Glutamine (Penstrep), Dulbecco’s Phosphate-Buffered Saline (PBS) and Non-Essential Amino Acids Solution (NEAA) were purchased from Fisher Scientific and Resazurin from ACROS Organics.

Instrumentation and methods

\(^1\)H and \(^{13}\)C NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer. Chemical shifts (\(\delta\)) are reported in parts per million (ppm) referenced to tetramethylsilane (\(\delta 0.00\)) ppm using the residual proton solvent peaks as internal standards. Coupling constants (\(J\)) are reported in Hertz (Hz) and the multiplicity is abbreviated as follows: s (singulet), d (doublet), dd (doublet of doublet), m (multiplet). ESI-MS experiments were carried out using a LTQ-Orbitrap XL from Thermo Scientific (Thermo Fisher Scientific) and operated in positive ionization mode, with a spray voltage at 3.6 kV. No Sheath and auxiliary gas was used. Applied voltages were 40 and 100 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 275°C. Detection was achieved in the Orbitrap with a resolution set to 100,000 (at m/z 400) and a m/z range between 150-2000 in profile mode. Spectrum was analyzed using the acquisition software XCalibur 2.1 (Thermo Fisher Scientific). The automatic gain control (AGC) allowed accumulation of up to \(2 \times 10^5\) ions for FTMS scans, Maximum injection time was set to 300 ms and 1 µscan was acquired. 10 µL was injected using a Thermo Finnigan Surveyor HPLC system (Thermo Fisher Scientific) with a continuous infusion of methanol at 100 µL.min\(^{-1}\). Elemental microanalyses were performed on a Thermo Flash 2000 elemental analyser. For analytic HPLC the following system has been used: 2 x Agilent G1361 1260 Prep Pump system with Agilent G7115A 1260 DAD WR Detector equipped with an Agilent Pursuit XRs 5C18 (100Å, C18 5 µm 250 x 4.6 mm) Column. The
solvents (HPLC grade) were millipore water (0.1% TFA, solvent A) and acetonitrile (0.1% TFA, solvent B). Inductive coupled plasma mass spectrometry (ICP-MS) experiments were carried out on an iCAP RQ ICP-MS instrument (Thermo Fisher).

**Synthesis**

[Ru(phen)$_2$($E,E'$)-4,4'$-Bis[p-(N,N-dimethylamino)styryl]-2,2'$-bipyridine)]$[PF_6]_2$ (1)

Ru(phen)$_2$Cl$_2$ (455 mg, 0.86 mmol, 1.0 equiv.) and ($E,E'$)-4,4'$-Bis[p-(N,N-dimethylamino)styryl]-2,2'$-bipyridine (458 mg, 1.03 mmol, 1.2 equiv.) were suspended in dry Ethanol (50 mL) under nitrogen atmosphere and the mixture was refluxed for 19 h. Then the solution was cooled down, undissolved residue was removed via filtration and washed with Ethanol. To the residual solution a sat. aqueous solution of NH$_4$PF$_6$ was added. The crude product, which precipitated as a PF$_6$ salt was collected by filtration and washed with water and Et$_2$O. The solid was dissolved in Dichloromethane and washed with a 5% LiCl aqueous solution, brine and water. The solvent was removed under reduced pressure and the product was purified via fractionated precipitation from Acetonitrile by adding dropwise Et$_2$O. The obtained solid was separated by filtration and was washed with H$_2$O, Et$_2$O and Pentane. 427 mg of 1 (0.36 mmol, 41%) were yielded as a red solid. $^1$H-NMR (CD$_3$CN, 400 MHz): $\delta$ = 8.68-8.63 (m, 4H), 8.54 (dd, $J = 8.3$, 1.4 Hz, 2H), 8.34-8.31 (m, 2H), 8.29-8.14 (m, 6H), 7.89 (dd, $J = 5.4$, 1.4 Hz, 2H), 7.81 (dd, $J = 8.3$, 5.1 Hz, 2H), 7.64 (d, $J = 16.4$ Hz, 2H), 7.57-7.49 (m, 4H), 7.46 (d, $J = 6.1$ Hz, 2H), 7.24 (dd, $J = 6.0$, 1.7 Hz, 2H), 7.00 (d, $J = 16.4$ Hz, 2H), 6.80-6.74 (m, 4H), 3.00 (s, 12H). $^{13}$C-NMR (CD$_3$CN, 100 MHz): $\delta$ = 158.3, 153.7, 153.6, 152.7, 152.4, 148.9, 148.7, 137.9, 137.6, 137.5, 132.0, 130.0, 129.0, 127.0, 126.8, 124.3, 124.2, 120.7, 119.5, 113.7, 113.1, 40.4. HR-MS (pos. detection mode): calcd for C$_{54}$H$_{46}$N$_8$Ru m/z [M]$^{2+}$ 454.1439; found: 454.1455. Elemental analysis calcd for C$_{54}$H$_{46}$F$_{12}$N$_8$P$_2$Ru (%): C 54.21, H 3.91, N 9.24; found: C 54.14, H 3.87, N 9.35.

[Ru(phen)$_2$($E,E'$)-4,4'$-Bis[p-methoxystyryl]-2,2'$-bipyridine)]$[PF_6]_2$ (2)

Ru(phen)$_2$Cl$_2$ (443 mg, 0.83 mmol, 1.0 equiv.) and ($E,E'$)-4,4'$-Bis[p-methoxystyryl]-2,2'$-bipyridine (420 mg, 0.99 mmol, 1.2 equiv.) were suspended in dry Ethanol (50 mL) under nitrogen atmosphere and the mixture was refluxed for 24 h. Then the solution was cooled down and undissolved residue was removed via filtration. To the residual solution a sat. aqueous
solution of NH₄PF₆ was added. The crude product, which precipitated as a PF₆ salt was collected by filtration and washed with water and Et₂O. The product was isolated by column chromatography on silica gel with an Acetonitrile/aq. KNO₃ (0.4 M) solution (10:1). The fractions containing the product were united and the solvent was removed under reduced pressure. The residue was dissolved in Acetonitrile and undissolved KNO₃ was removed by filtration. The solvent was removed again and the product was dissolved in H₂O (50 mL). Upon addition of NH₄PF₆ the product precipitated as a PF₆ salt. The solid was obtained by filtration and was washed three-times with H₂O and Et₂O. 672 mg of 2 (0.57 mmol, 69 %) were yielded as a red solid. ¹H-NMR (CD₃CN, 400 MHz): δ = 8.70 (s, 2H), 8.65 (d, J = 8.2 Hz, 2H), 8.55 (d, J = 8.2 Hz, 2H), 8.32-8.22 (m, 6H), 7.89 (d, J = 4.7 Hz, 2H), 7.81 (dd, J = 8.2, 5.2 Hz, 2H), 7.68 (d, J = 16.2 Hz, 2H), 7.63-7.53 (m, 8H), 7.30 (d, J = 5.5 Hz, 2H), 7.13 (d, J = 16.2 Hz, 2H), 7.01 (d, J = 8.0 Hz, 4H), 3.83 (s, 6H). ¹³C-NMR (CD₃CN, 100 MHz): δ = 162.0, 158.4, 153.7, 153.6, 152.7, 148.9, 148.6, 148.1, 137.7, 137.6, 137.0, 132.0, 129.4, 129.0, 127.0, 126.8, 124.7, 122.6, 121.2, 115.5, 56.1. HR-MS (pos. detection mode): calcd for C₅₂H₄₀N₆O₂Ru m/z [M]+ 441.1123; found: 441.1131. Elemental analysis calcd for C₅₂H₄₀F₁₂N₆O₂P₂Ru (%): C 53.29, H 3.44, N 7.17; found: C 53.18, H 3.35, N 7.26. [Ru(phen)₂((E,E')-4,4´-Bis[p-(N,N-methoxy)styryl]-2,2´-bipyridine)][Cl]₂: The counter ion PF₆ was exchanged to Cl by elution with MeOH from the ion exchange resin Amberlite IRA-410. Elemental analysis calcd for C₅₂H₄₀N₆O₂RuCl₂ (%): C 65.54, H 4.23, N 8.82; found: C 65.37, H 4.11, N 8.60.

[Ru(4,7-Diphenyl-1,10-phenanthroline)₂((E,E')-4,4´-Bis[p-(N,N-dimethylamino)styryl]-2,2´-bipyridine)][PF₆]₂ (3)

Ru(4,7-Diphenyl-1,10-phenanthroline)Cl₂ (335 mg, 0.40 mmol, 1.0 equiv.) and (E,E')-4,4´-Bis[p-(N,N-dimethylamino)styryl]-2,2´-bipyridine (215 mg, 0.48 mmol, 1.2 equiv.) were suspended in dry Ethanol (100 mL) under nitrogen atmosphere and the mixture was refluxed for 24 h. Then the solution was cooled down and undissolved residue was removed via filtration. The residual solution was diluted with a mixture of Ethanol and water and a sat. aqueous solution of NH₄PF₆ was added. The crude product, which precipitated as a PF₆ salt was collected by centrifugation and washed with water and Et₂O. The solid was dissolved in Acetonitrile and undissolved residue was removed via filtration. The solvent was removed under reduced pressure and the obtained solid was dissolved in Dichloromethane and washed with a 5% LiCl aqueous solution, brine and water. The solvent was removed under reduced pressure. The product was isolated via fractionated precipitation from Methanol by adding
dropwise Et₂O. After drying, 289 mg of 3 (0.19 mmol, 48 %) were yielded as a red solid. ¹H-
NMR (CD₃CN, 400 MHz): δ = 8.73 (d, J = 1.5 Hz, 2H), 8.42 (d, J = 5.5 Hz, 2H), 8.26 – 8.16
(m, 4H), 8.12 (d, J = 5.5 Hz, 2H), 7.78 (d, J = 5.5 Hz, 2H), 7.70 (d, J = 16.3 Hz, 2H), 7.67 –
7.57 (m, 24H), 7.54 (d, J = 8.9 Hz, 4H), 7.34 (dd, J = 6.1, 1.7 Hz, 2H), 7.05 (d, J = 16.3 Hz,
2H), 6.78 (d, J = 9.0 Hz, 4H), 3.01 (s, 12H). ¹³C-NMR (CD₃CN, 100 MHz): δ = 158.3, 153.1,
152.7, 152.4, 149.9, 149.8, 149.5, 149.4, 148.8, 138.0, 136.8, 136.7, 130.8, 130.8, 130.6, 130.1,
130.1, 130.0, 129.9, 127.2, 127.0, 127.0, 127.0, 124.3, 120.8, 119.5, 113.1, 40.4. HR-MS (pos.
detection mode): calcd for C₇₈H₆₂N₈Ru m/z [M]²⁺ 606.2065; found: 606.2078. Elemental
analysis calcd for C₇₈H₆₂N₈RuP₂F₁₂ + 1.5 CH₃OH (%): C 61.59, H 4.42, N 7.23; found: C 61.73,
H 4.48, N 6.88.

[Ru(4,7-Diphenyl-1,10-phenanthroline)₂((E,E')-4,4’-Bis[p-methoxystyryl]-2,2’-
bipyridine)][PF₆]₂ (4)

Ru(4,7-Diphenyl-1,10-phenanthroline)₂Cl₂ (300 mg, 0.36 mmol, 1.0 equiv.) and (E,E')-4,4´-
Bis[p-methoxystyryl]-2,2´-bipyridine (181 mg, 0.43 mmol, 1.2 equiv.) were suspended in dry
Ethanol (50 mL) under nitrogen atmosphere and the mixture was refluxed for 24 h. Then the
solution was cooled down and undissolved residue was removed via filtration. The residual
solution was diluted with a mixture of Ethanol and water and a sat. aqueous solution of NH₄PF₆
was added. The crude product, which precipitated as a PF₆ salt was collected by filtration and
washed with water and Et₂O. The solid was dissolved in Dichloromethane and washed with a
5% LiCl aqueous solution, brine and water. The solvent was removed under reduced pressure.
After drying, 395 mg of 4 (0.27 mmol, 74 %) were yielded as a red solid. ¹H-NMR (CD₃CN,
400 MHz): δ = 8.78 (s, 2H), 8.41 (d, J = 5.5 Hz, 2H), 8.21 (s, 4H), 8.13 (d, J = 5.5 Hz, 2H),
7.78 (d, J = 5.5 Hz, 2H), 7.75 (d, J = 2.3 Hz, 2H), 7.72 (d, J = 8.0 Hz, 2H), 7.66-7.58 (m, 26H),
7.41 (d, J = 6.1 Hz, 2H), 7.19 (d, J = 16.4 Hz, 2H), 7.02 (d, J = 8.7 Hz, 4H), 3.84 (s, 6H). ¹³C-
NMR (CD₃CN, 100 MHz): δ = 162.0, 158.4, 153.1, 152.8, 150.0, 149.9, 149.5, 149.3, 148.2,
137.1, 136.8, 136.7, 130.8, 130.8, 130.7, 130.7, 130.1, 130.1, 130.1, 130.0, 129.9, 129.4, 127.2, 127.0,
124.9, 122.7, 121.3, 115.5, 56.2. HR-MS (pos. detection mode): calcd for C₇₆H₅₆N₆O₂Ru m/z [M]²⁺ 593.1749; found: 593.1768. Elemental analysis calcd for C₇₆H₅₆F₁₂N₆O₂P₂Ru + 4 H₂O
(%): C 58.95, H 4.17, N 5.43; found: C 58.78, H 3.94, N 5.72.
[Ru(1,10-phenanthroline)₃][PF₆]₂

[Ru(phen)₃][PF₆]₂ was synthesized as previously published using RuCl₂(dms)₄ precursor.[³]
Purity of the sample was assessed by HPLC and elemental analysis. Elemental analysis calcd for C₃₆H₂₄F₁₂N₆P₂Ru (%): C 46.41, H 2.60, N 9.02; found: C 46.34, H 2.54, N 8.83.

[Ru(4,7-Diphenyl-1,10-phenanthroline)₃][PF₆]₂

[Ru(bphen)₃][PF₆]₂ was synthesized as previously published[⁴] using RuCl₂(dms)₄ precursor. Purity of the sample was assessed by HPLC and elemental analysis. Elemental analysis calcd for C₇₂H₄₈F₁₂N₆P₂Ru (%): C 62.30, H 3.49, N 6.05; found: C 62.28, H 3.44, N 5.92.

Spectroscopic measurements

The absorption of the samples has been measured with a SpectraMax M2 Spectrometer (Molecular Devices). The emission was measured by irradiation of the sample in fluorescence quartz cuvettes (width 1 cm) using a NT342B Nd-YAG pumped optical parametric oscillator (Ekspla) at 355 nm. Luminescence was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. As a detector a XPI-Max 4 CCD camera (Princeton Instruments) has been used.

Two-photon absorption cross-section measurements

The two-photon absorption of the samples was determined using the two-photon induced luminescence method relative to Rhodamine B. The data was measured with an OpoletteTM 355II (pulse width ≤ 100 fs, 80 MHz repetition rate, Spectra Physics Inc.). The experimental luminescence excitation and detection conditions were conducted with negligible reabsorption processes, which can affect TPA measurements. The quadratic dependence of two-photon induced luminescence intensity on the excitation power was verified at an excitation wavelength of 800 nm. The two-photon absorption cross section of the probes was using the following equation:

\[
\sigma_{2, \text{sample}} = \sigma_{2, \text{reference}} \times (\phi_{\text{reference}} \times c_{\text{reference}} \times I_{\text{sample}} \times n_{\text{sample}}) / (\phi_{\text{sample}} \times c_{\text{sample}} \times I_{\text{reference}} \times n_{\text{reference}})
\]
σ₂ = two photon cross-section, φ = quantum yield, c = concentration, I = integrated luminescence intensity, n = refractive index

### Luminescence quantum yield measurements

For the determination of the luminescence quantum yield, the samples were prepared in an CH₃CN solution with an absorbance of 0.1 at 355 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a NT342B Nd-YAG pumped optical parametric oscillator (Ekspla) at 355 nm. The emission signal was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. As a detector a XPI-Max 4 CCD camera (Princeton Instruments) has been used. The luminescence quantum yields were determined by comparison with the reference [Ru(bipy)₃]Cl₂ in CH₃CN (Φₑm=5.9%)⁵¹ applying the following formula:

$$\Phi_{ₑm, \text{sample}} = \Phi_{ₑm, \text{reference}} \times \left( \frac{F_{\text{reference}}}{F_{\text{sample}}} \right) \times \left( \frac{I_{\text{sample}}}{I_{\text{reference}}} \right) \times \left( \frac{n_{\text{sample}}}{n_{\text{reference}}} \right)^2$$

F = 1 – 10⁻ᴬ

Φₑm = luminescence quantum yield, F = fraction of light absorbed, I = integrated emission intensities, n = refractive index, A = absorbance of the sample at irradiation wavelength.

### Lifetime measurements

For the determination of the lifetimes, the samples were prepared in an air saturated and in a degassed CH₃CN solution with an absorbance of 0.2 at 355 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a NT342B Nd-YAG pumped optical parametric oscillator (Ekspla) at 355 nm. The emission signal was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. As a detector a R928 photomultiplier tube (Hamamatsu) has been used.

### Electron spin resonance measurements

For verification of the reactive species formed upon light exposure of the compounds, the respective ESR spectra were recorded. The samples with a final concentration of 10 μM were dissolved in CH₃CN or PBS containing 20 mM TEMP (2,2,6,6-tetramethylpiperidine) as a ¹O₂ scavenger or 20 mM DMPO (5,5-dimethyl-1-pyroline N-oxide) as a *OH radical scavenger.
Capillary tubes were filled with the solution and sintered by fire. EPR spectra were recorded on a Bruker A300 spectrometer with 1 G field modulation, 100 G scan range and 20 mW microwave power. The samples were measured in exclusion from light and after irradiation for 60 s (450 ± 10 nm, 21.8 mW cm⁻²).

Singlet oxygen quantum yield measurements

- Direct evaluation

The samples were prepared in an air saturated CH₃CN or D₂O solution with an absorbance of 0.2 at 450 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a mounted M450LP1 LED (Thorlabs) whose irradiation, centered at 450 nm, has been focused with aspheric condenser lenses. The intensity of the irradiation has been varied using a T-Cube LED Driver (Thorlabs) and measured with an optical power and energy meter. The emission signal was focused and collected at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. A longpass glass filter was placed in front of the monochromator entrance slit to cut off light at wavelengths shorter than 850 nm. As a detector an EO-817L IR-sensitive liquid nitrogen cooled germanium diode detector (North Coast Scientific Corp.) has been used. The singlet oxygen luminesce at 1270 nm was measured by recording spectra from 1100 to 1400 nm. For the data analysis, the singlet oxygen luminescence peaks at different irradiation intensities were integrated. The resulting areas were plotted against the percentage of the irradiation intensity and the slope of the linear regression calculated. The absorbance of the sample was corrected with an absorbance correction factor. As reference for the measurement Rose Bengal (Φ = 76%)[6] was used and the singlet oxygen quantum yields were calculated using the following formula:

\[
\Phi_{\text{sample}} = \Phi_{\text{reference}} \times \left( \frac{S_{\text{sample}}}{S_{\text{reference}}} \right) \times \left( \frac{I_{\text{reference}}}{I_{\text{sample}}} \right)
\]

\[
I = I_0 \times (1 - 10^{-A})
\]

Φ = singlet oxygen quantum yield, S = slope of the linear regression of the plot of the areas of the singlet oxygen luminescence peaks against the irradiation intensity, I = absorbance correction factor, I₀ = light intensity of the irradiation source, A = absorbance of the sample at irradiation wavelength.
- **Indirect evaluation**

For the measurement in CH$_3$CN: The samples were prepared in an air-saturated CH$_3$CN solution containing the complex with an absorbance of 0.2 at the irradiation wavelength, $N,N$-dimethyl-4-nitrosoaniline aniline (RNO, 24 µM) and imidazole (12 mM). For the measurement in PBS buffer: The samples were prepared in an air-saturated PBS solution containing the complex with an absorbance of 0.1 at the irradiation wavelength, $N,N$-dimethyl-4-nitrosoaniline aniline (RNO, 20 µM) and histidine (10 mM). The samples were irradiated on 96 well plates with an Atlas Photonics LUMOS BIO irradiator for different times. The absorbance of the samples was measured during these time intervals with a SpectraMax M2 Microplate Reader (Molecular Devices). The difference in absorbance ($A_0$-$A$) at 420 nm for the CH$_3$CN solution or at 440 nm a PBS buffer solution was calculated and plotted against the irradiation times. From the plot the slope of the linear regression was calculated as well as the absorbance correction factor determined. The singlet oxygen quantum yields were calculated using the same formulas as used for the direct evaluation.

**Stability in human plasma**

The stability of the complexes was evaluated with caffeine as an internal standard, which has already been shown to be suitable for these experiments.""}[7]\] The pooled human plasma was obtained from Biowest and caffeine from TCI Chemicals. Stock Solutions of the compounds (40 µM) and caffeine (40 µM) were prepared in DMSO. One aliquot of the solutions was added to 975 µL of human plasma to a total volume of 1000 µL. Final concentrations of the compounds of 0.25 µM and caffeine of 0.5 µM were achieved. The resulting solution was incubated for 48 h at 37 °C with continuous gentle shaking (ca. 300 rpm). The reaction was stopped after the incubation time by addition of 3 mL of methanol. The mixture was centrifuged for 60 min at 3000 rpm at 4 °C. The methanolic solution was filtered through a 0.2 µm membrane filter. The solvent was evaporated under reduced pressure and the residue was dissolved in 1:1 (v/v) CH$_3$CN/ H$_2$O 0.1% TFA solution. The solution was filtered through a 0.2 µm membrane filter and analyzed using a HPLC System. The solvents (HPLC grade) were millipore water (0.1% TFA, solvent A) and acetonitrile (solvent B). Method M1: 0-3 minutes: isocratic 95% A (5% B); 3-17 minutes: linear gradient from 95% A (5% B) to 0% A (100% B); 17-23 minutes: isocratic 0% A (100% B); Method M2: 0-3 minutes: isocratic 50% A (50% B); 3-17 minutes: linear gradient from 50% A (50% B) to 0% A (100% B). The flow rate was 1 mL/min and the chromatogram was detected at 250nm.
Photostability

The samples were prepared in an air saturated CH$_3$CN solution with an absorbance of about 0.5 at 450 nm. To measure the photostability, the samples were irradiated at 540 nm in 96 well plates with an Atlas Photonics LUMOS BIO irradiator during time intervals from 0-30 min. The absorbance spectrum from 250-650 nm was recorded with a SpectraMax M2 Microplate Reader (Molecular Devices) after each time interval and compared.

Cell culture

The human glioblastoma astrocytoma (U373) cell line was cultured in MEM medium supplemented with 10% FBS, 1% NEAA (non-essential amino-acids) and 1% penicillin/streptomycin. The human cervical carcinoma (HeLa), the mouse colon carcinoma (CT-26) and adenocarcinomic human alveolar basal epithelial (A549) cell lines were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The human retinal epithelial cells (RPE-1, non-cancerous cells immortalized with hTERT) were cultured in DMEM-F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were obtained from the American Type Culture Collection (ATCC) and cultured at 37°C and 5% CO$_2$. Before an experiment, the cells were passaged three times.

Time dependent cellular uptake

The cellular uptake of the compound was investigated by the determination of the Ru content inside the cells after different incubation times. The compound with a final concentration of 10 μM (2% DMSO, v%) was incubated for varying times (0.5, 1, 2, 4, 6, 8, 12 h) at 37 °C on a cell culture dish with a density of ca. 4-6*10$^6$ cells in 10 mL of media. After this time, the media was removed and the cells washed with cell media. The cells were trypsinised, harvested, centrifuged and resuspended. The number of cells on each dish was accurately counted. Each sample was the digested using a 60% HNO$_3$ solution for three days. Each sample was diluted to solution of 2% HNO$_3$ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.
**Distribution coefficient**

The lipophilicity of a complex was determined by measuring its distribution coefficient between the PBS and Octanol phase by using the “shake-flask” method. For this technique, the used phases were previously saturated in each other. The complex was dissolved in the phase (A) with its major presence. This solution was then mixed with an equal volume of the other phase (B) at 80 rpm for 8 h and then equilibrated overnight. The phase A was then carefully separated from phase B. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The evaluation of the complexes was repeated three times and the ratio between the organic and aqueous phase calculated.

**Cellular uptake mechanism**

The mechanism of the cellular uptake was investigated by systematic inhibition of different uptake pathways and afterwards determination of the amount of Ru inside the cells via ICP. For the experiment, 10^6 cells were pretreated with the corresponding inhibitor.

Control: The cells were incubated with the compound (10 μM, 2% DMSO, v%) for 1 h at 37°C. After this time, the cells were washed with PBS. The cells were detached with trypsin, harvested, centrifuged and resuspended. The number of cells on each dish was accurately counted. Each sample was digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.

Low temperature: The cells were incubated at 4°C for 1 h. The cells were further incubated with the compound (10 μM, 2% DMSO, v%) for 1 h at 4°C. After this time, the cells were washed with PBS. The cells were detached with trypsin, harvested, centrifuged and resuspended. The number of cells on each dish was accurately counted. Each sample was digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.

Metabolic Inhibition: The cells were incubated with 2-Deoxy-D-glucose (50 mM) and oligomycin (5 μM) for 1 h. After the preincubation, the cells were washed with PBS and further incubated with the compound (10 μM, 2% DMSO, v%) for 1 h at 37°C. After this time, the
cells were washed with PBS. The cells were detached with trypsin, harvested, centrifuged and resuspended. The number of cells on each dish was accurately counted. Each sample was digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.

Endocytic inhibition: The cells were incubated with NH₄Cl (50 mM) or chloroquine (100 μM) for 1h. After the preincubation, the cells were washed with PBS and further incubated with the compound (10 μM, 2% DMSO, v%) for 1 h at 37°C. After this time, the cells were washed with PBS. The cells were detached with trypsin, harvested, centrifuged and resuspended. The number of cells on each dish was accurately counted. Each sample was digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.

Cation transporter inhibition: The cells were incubated with tetraethylammonium chloride (1 mM) for 1h. After the preincubation, the cells were washed with PBS and further incubated with the compound (10 μM, 2% DMSO, v%) for 1 h at 37°C. After this time, the cells were washed with PBS. The cells were detached with trypsin, harvested, centrifuged and resuspended. The number of cells on each dish was accurately counted. Each sample was digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.

Intracellular distribution by confocal luminescence imaging

The co-localisation of the compound was determined under usage of its luminescence properties. 10⁴ cells were seeded on 35 mm confocal dishes and allowed to adhere overnight. The cells were incubated with the compound (10 μM, 2% DMSO, v%) for 8 h at 37°C in the dark. After this time, the cells were washed with PBS. The cells were further each independently incubated with LysoTracker Green, MitoTracker Deep Red, Cell-light Golgi-GFP, ER tracker Green and Hoechst 33342 for 30 min at 37 °C in the dark. The cells were washed three times with PBS. Confocal images were taken with a 63x oil-immersion objective lens by an LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with Argon and a HeNe laser and a Coherent Chameleon 2-Photon laser and a GaAsP detector. The organelle trackers MitoTracker
Deep Red (MTR, $\lambda_{ex} = 633$ nm, $\lambda_{em} = 650 - 720$ nm), LysoTracker Green (LTG, $\lambda_{ex} = 488$ nm, $\lambda_{em} = 490 - 550$ nm) and Hoechst 33342 (Hoechst, $\lambda_{ex} = 405$ nm, $\lambda_{em} = 410 - 470$ nm) were excited and detected as recommended by the supplier. The investigated Ru complexes were detected under usage of their 1-Photon ($\lambda_{ex} = 458$ nm, $\lambda_{em} = 600 - 750$ nm) and 2-Photon ($\lambda_{ex} = 800$ nm, $\lambda_{em} = 600 - 750$ nm) luminescence properties.

**Intracellular distribution by ICP-MS**

The co-localisation of the compound was determined by measuring the Ru content inside the cell via ICP-MS. $10^6$ cells were incubated with the compound (10 $\mu$M, 2% DMSO, $\nu$%) for 6 h at 37°C in the dark. After this time, the cells were detached with trypsin and harvested. The number of cells was accurately counted. The amount was equally divided into four portions. In the first portion, the nucleus was extracted using a nucleus extraction kit (Sangon Biotech); in the second portion, the mitochondria was extracted using a mitochondria extraction kit (Sangon Biotech) and in the third portion, the lysosome was extracted using a lysosome extraction kit (GenMed Scientific). Using the fourth portion, the cytoplasm was extracted. For this, the cells were detached with trypsin, harvested and centrifuged. The cell pellet was resuspended in lysis buffer and the cells were lysed. The cell organelles were separated using a vacuum ultracentrifuge (Optima MAX-XP ultracentrifuge, Beckman Coulter) for 150 min at 200000 g at 4 °C. The supernatant solution was separated. Each sample was the digested using a 60% HNO$_3$ solution for three days and was diluted to a solution of 2% HNO$_3$ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references. The Ru content was then associated with the number of cells.

**(Photo-)cytotoxicity on 2D cell monolayers**

The cytotoxicity of the compounds was assessed by measuring cell viability using a fluorometric resazurin assay. The cultivated cells were seeded in triplicates in 96 well plates with a density of 4000 cells per well in 100 $\mu$L of media. After 24 h, the medium was removed and the cells were treated with increasing concentrations of the compound diluted in cell media achieving a total volume of 200 $\mu$L. The cells were incubated with the compound for 4 h. After this time, the media was removed and replaced with 200 $\mu$L of fresh medium. For the phototoxicity studies, the cells were exposed to light with an Atlas Photonics LUMOS BIO
irradiator. Each well was constantly illuminated with either a 480 nm (spectral half-width: 20 nm, 10 min, 3.1 J/cm²), 540 nm (spectral half-width: 30 nm, 40 min, 9.5 J/cm²) or 595 nm (spectral half-width: 20 nm, 60 min, 11.2 J/cm²) irradiation. During this time, the temperature was maintained constantly at 37 °C. The cells were grown in the incubator for additional 44 h. For the determination of the dark cytotoxicity, the cells were not irradiated and after the medium exchange directly incubated for 44 h. After this time, the medium was replaced with fresh medium containing resazurin with a final concentration of 0.2 mg/mL. After 4 h incubation, the amount of the fluorescent product resorufin was determined upon excitation at 540 nm and measurement its emission at 590 nm using a SpectraMax M2 Microplate Reader (Molecular Devices). The obtained data was analyzed with the GraphPad Prism software.

Cell death mechanism

The cell death mechanism assay was investigated by measuring the cell viability using a fluorometric resazurin assay after preincubation with various cell death inhibitors. 3-methyladenine (100 μM), Z-VAD-FMK (20 μM), cycloheximide (0.1 μM) and necrostatin-1 (60 μM) were pre-incubated in Hela cells for 40 min. The compounds were then incubated with the IC₅₀ concentration for 4 h. After this time, the media was removed and replaced with 200 μL of fresh medium. For the phototoxicity studies, the cells were exposed to light with an Atlas Photonics LUMOS BIO irradiator. Each well was constantly illuminated with a 480 nm irradiation. During this time, the temperature was maintained constantly at 37 °C. The cells were grown in the incubator for additional 44 h. For the determination of the dark cytotoxicity, the cells were not irradiated and after the medium exchange directly incubated for 44 h. After this time, the medium was replaced with fresh medium containing resazurin with a final concentration of 0.2 mg/mL. After 4 h incubation, the amount of the fluorescent product resorufin was determined upon excitation at 540 nm and measurement its emission at 590 nm using a SpectraMax M2 Microplate Reader (Molecular Devices). The obtained data was analyzed with the GraphPad Prism software.

Caspase-3/7 activation

Caspase-3/7 activity was measured using Caspase-Glo-3/7 assay kit (Promega). The cultivated cells were seeded in triplicates in 96 well plates with a density of 4000 cells per well in 100 μL
of media. After 24 h, the medium was removed and the cells were treated with half of the IC$_{50}$ concentrations of the compound diluted in cell media achieving a total volume of 200 μL. For the phototoxicity studies, the cells were exposed to light with an Atlas Photonics LUMOS BIO irradiator. Each well was constantly illuminated with either a 480 nm irradiation. During this time, the temperature was maintained constantly at 37 °C. The cells were grown in the incubator for additional 12 h. After this time, Caspase-Glo 3/7 reagent (100 μL) was added and the cells incubated for an additional 1 h in the dark. The generated chemiluminescence was measured using a SpectraMax M2 Microplate Reader (Molecular Devices).

**Generation and analysis of 3D multicellular tumour spheroids (MCTS)**

A suspension of 0.75% agarose in PBS buffer was heated inside a high-pressure autoclave. The hot emulsion was transferred into wells (50 μL per well) of a 96 cell culture well plate. The plates were exposed for 3h to UV irradiation to ensure the sterility and allow the agarose solution to cool down. After this time, the agarose was overlayed with a HeLa cell suspension at a density of 3000 cells per well in 150 μL of media. The MCTS were cultivated and maintained at 37 °C in a cell culture incubator at 37 °C with 5% CO$_2$ atmosphere. The culture media was replaced every two days. Within two-three days MCTS were formed from the cell suspension. The formation as well as integrity, diameter and volume of the MCTS was monitored by an Axio Observer Z1 (Carl Zeiss) phase contrast microscope. The volume was calculated using the following formula: $V = \frac{4}{3} \pi r^3$. The luminescence images along the z-axis were captured by a one- ($\lambda_{ex} = 458$ nm, $\lambda_{em} = 600 - 750$ nm) or two-photon ($\lambda_{ex} = 830$ nm, $\lambda_{em} = 600 - 750$ nm) excitation in the z-stack mode with a an LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with Argon and a Coherent Chameleon 2-Photon laser and a GaAsP detector.

**Irradiation induced cellular singlet oxygen generation in 3D MCTS**

The MCTSs were treated with the compound (2 μM, 2% DMSO, v%) for 12 h in the dark, the culture medium was removed and PBS containing DCFH-DA (20.0 mM) was incubated for 30 min. The solution was removed, the cells washed and fresh PBS added. The MCTS were exposed to a 2P irradiation (800 nm, 2 J/cm$^2$, section interval of 5 μm). Confocal luminescence
images ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 510 - 550$ nm) were taken with a LSM 810 (Carl Zeiss) laser scanning confocal microscope before and after the irradiation.

**3D multicellular tumour spheroids (MCTS) growth inhibition assay**

MCTS were treated with the corresponding compounds (20 $\mu$M, 20 $\mu$M tetraphenylporphyrin H$_2$TPP, 10 $\mu$M cisplatin, 30 $\mu$M cisplatin, 2% DMSO, $\nu$%) by replacing 50% of the media with drug supplemented media in the dark for 12 h. After this time, the MCTS were exposed to a one-photon (500 nm, 10 J/cm$^2$) using a LED light or a two-photon irradiation (800 nm, 10 J/cm$^2$) with a section interval of 5 μm using a LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with a Coherent Chameleon 2-Photon laser. The cell culture media was replaced every two days. The integrity and diameter of the MCTs was monitored with an Axio Observer Z1 (Carl Zeiss) phase contrast microscope every 24 h.

**3D multicellular tumour spheroids (MCTS) viability assay**

MCTS were treated with the corresponding compounds (20 $\mu$M, 2% DMSO, $\nu$%) by replacing 50% of the media with drug supplemented media in the dark for three days. After this time, the MCTS were exposed to a one-photon (500 nm, 10 J/cm$^2$) or two-photon irradiation (800 nm, 10 J/cm$^2$) with a section interval of 5 μm using a LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with a Coherent Chameleon 2-Photon laser. The cell culture media was replaced every two days. Two days after the irradiation the MCTS viability was tested using a Viability/Cytotoxicity Kit for mammalian cells (Invitrogen). The MCTS were stained with calcein-AM ($\lambda_{\text{ex}} = 490$ nm, $\lambda_{\text{em}} = 515$ nm) and propidium iodide ($\lambda_{\text{ex}} = 536$ nm, $\lambda_{\text{em}} = 617$ nm) and their luminescence images taken with an Axio Observer Z1 (Carl Zeiss, Germany) inverted fluorescence microscope.

**(Photo-)cytotoxicity in 3D multicellular tumour spheroids (MCTS)**

The cytotoxicity of the compounds in 3D multicellular tumour spheroids (MCTS) was assessed by measurement of the ATP concentration. MCTS were treated with increasing concentrations of the compound (2% DMSO, $\nu$%) by replacing 50% of the media with drug supplemented media and incubation for 12 h. After this time, the MCTS were divided in three identical groups.
The first group was strictly kept in the dark. The second group was exposed to a one-photon irradiation (500 nm, 10 J/cm²) using a LED and the third group was exposed to a two-photon irradiation (800 nm, 10 J/cm²) with a section interval of 5 μm using a LSM 880 (Carl Zeiss, Germany) laser scanning confocal microscope equipped with a Coherent Chameleon 2-Photon laser. After the irradiation, all groups were incubated additional 48 h. The ATP concentration was measured using a CellTiter-Glo 3D Cell Viability kit (Promega) by measuring the generated chemiluminescence with an infinite M200 PRO (Tecan) plate reader. The obtained data was analyzed with the GraphPad Prism software.

**In vivo tumour xenograft**

6×10⁶ A549 cells were subcutaneously (s.c.) injected in the nude mice as a cell suspension in 150 μL Matrigel (Corning) and saline (1:1, v/v). After a week, the tumour volumes of the mice reached approximately 80 mm³.

**In vivo biodistribution**

Two nude mice were tail-intravenous (i.v.) injected with the compound (0.5 mg/Kg). After 2h, the mice were sacrificed and the major organs (including the heart, liver, spleen, lung, kidney, brain, intestine and blood) were separated, weighted and grinded. The samples were digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references.

**In vivo time-dependent biodistribution**

12 A549 tumour-bearing nude mice were separated into 4 groups and i.v. injected with the compound (0.5 mg/Kg). After 30min, 1h, 2h and 4h, the mice were sacrificed and the major organs (including the heart, liver, spleen, lung, kidney, brain, intestine and blood) were separated, weighted and grinded. The samples were digested using a 60% HNO₃ solution for three days. Each sample was diluted to solution of 2% HNO₃ in water. The Ru content was determined using an ICP-MS apparatus and comparing the results with the Ru references.
In vivo PDT treatment

30 A549 tumour-bearing nude mice were randomly separated into 6 groups. The mice were treated on day 1, 4 and 7. After being anaesthetized, the nude mouse was fixed in the warm three-axes holder. After disinfection with alcohol, for 1P a LED area light source and for 2P a low power beam of light (Femtosecond Fluorescence Spectrum Measurement System, SR-500I-D1, Coherent Inc, 800 nm, 500 μW, 1 kHz, pulse width 35 fs,) was used at the tumour site. The tumour was separated into two slices and the depth of each layer is 2 mm. Next, the power of laser was increased to 50 mW. Each slice was exposure under the laser by a reciprocating linear scanning (5 s/mm, 50 s/slices). After that, the operative area was disinfection by iodophor.

Group 1: injected 50 μL physiological saline;

Group 2: intravenous injected 2 (0.5 mg/Kg, 50 μL);

Group 3: injected by physiological saline (50 μL) and treated with 500 nm light (10 mW/cm², 15 min) 2h after injection;

Group 4: injected 2 (0.5 mg/Kg 50 μL) intravenously and irradiate under 500 nm light (10 mW/cm², 15 min) 2h after injection;

Group 5: injected by physiological saline (50 μL) and treated with 800 nm laser (50 mW, 1 kHz, pulse width 35 fs, 5 s/mm) 2h after injection;

Group 6: injected 2 (0.5 mg/Kg 50 μL) intravenously and irradiate under 800 nm laser (50 mW, 1 kHz, pulse width 35 fs, 5 s/mm) 2h after injection.

The tumour volume and body weight were measured and recorded every two days. Tumour volume was calculated by the following formula:

\[ Volume = \frac{Length \times Width^2}{2} \]

Histological examination

On day 17, the tumour and major organs (including the heart, liver, spleen, lung, kidney, brain, intestine and tumour) were collected and fixated by 4% paraformaldehyde. Slices of the respective organs were prepared in paraffin and stained with hematoxylin and eosin (H&E). A Carl Zeiss Axio Imager Z2 microscope was used to observe the tissue structure and cell state of the sections.
**Scheme S1.** Synthesis of the Bisstyryl-2,2’-bipyridine based ligands. a) 4-(Dimethylamino)benzaldehyde, potassium tert-butoxide, DMF, 90°C, 19 h, nitrogen atmosphere; b) 4-Methoxybenzaldehyde, potassium tert-butoxide, DMF, RT, 24 h, nitrogen atmosphere.
**Scheme S2.** Synthesis of the Ru(II) complexes 1-4. a) 1,10-Phenanthroline, LiCl, DMF, reflux, overnight, nitrogen atmosphere; b) \((E,E')-4,4'-\text{Bis}[p-(N,N\text{-dimethylamino})\text{styryl}]-2,2'-\text{bipyridine} / (E,E')-4,4'-\text{Bis}[p-(N,N\text{-methoxy})\text{styryl}]-2,2'-\text{bipyridine}, \text{EtOH, overnight, nitrogen atmosphere}; c) 4,7-Diphenyl-1,10-phenanthroline, LiCl, DMF, reflux, overnight, nitrogen atmosphere; d) \((E,E')-4,4'-\text{Bis}[p-(N,N\text{-dimethylamino})\text{styryl}]-2,2'-\text{bipyridine} / (E,E')-4,4'-\text{Bis}[p-(N,N\text{-methoxy})\text{styryl}]-2,2'-\text{bipyridine}, \text{EtOH, overnight, nitrogen atmosphere.}
**Figure S1.** $^1$H NMR spectrum of 1 in CD$_3$CN, 400 MHz.

**Figure S2.** $^{13}$C NMR spectrum of 1 in CD$_3$CN, 100 MHz.
Figure S3. ESI-HRMS spectrum of 1 (positive detection mode).

Figure S4. $^1$H NMR spectrum of 2 in CD$_3$CN, 400 MHz.
Figure S5. $^{13}$C NMR spectrum of 2 in CD$_3$CN, 100 MHz.

Figure S6. ESI-HRMS spectrum of 2 (positive detection mode).
Figure S7. $^1$H NMR spectrum of 3 in CD$_3$CN, 400 MHz.

Figure S8. $^{13}$C NMR spectrum of 3 in CD$_3$CN, 100 MHz.
Figure S9. ESI-HRMS spectrum of 3 (positive detection mode).

Figure S10. $^1$H NMR spectrum of 4 in CD$_3$CN, 400 MHz.
Figure S11. $^{13}$C NMR spectrum of 4 in CD$_3$CN, 100 MHz.

Figure S12. ESI-HRMS spectrum of 4 (positive detection mode).
Figure S13. a) 1P and b) 2P absorption spectra of 1-4.
**Table S1.** Spectroscopic properties of the complexes 1-4 in CH$_3$CN at room temperature. $\lambda_{\text{abs}}$ = Absorption maximum, $\lambda_{\text{em}}$ = Emission maximum, $\Phi_{\text{em}}$ = Luminenscene Quantum Yield, $\tau$ = Lifetime.

<table>
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<th>Compound</th>
<th>$\lambda_{\text{abs}}$ / nm ($\varepsilon$ / M$^{-1}$ cm$^{-1}$ × 10$^3$)</th>
<th>$\lambda_{\text{em}}$ / nm</th>
<th>$\Phi_{\text{em}}$ / %</th>
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**Figure S14.** Normalized emission spectra of the complexes 1-4 in CH$_3$CN.
Figure S15. Lifetime spectra of the complex 1 in aerated (above) and degassed (below) CH$_3$CN.
Figure S16. Lifetime spectra of the complex 2 in aerated (above) and degassed (below) CH$_3$CN.
Figure S17. Lifetime spectra of the complex 3 in aerated (above) and degassed (below) CH$_3$CN.
Figure S18. Lifetime spectra of the complex 4 in aerated (above) and degassed (below) CH$_3$CN.
Figure S19. ESR spectra of the complex 1 trapped by TEMP in CH$_3$CN (above) and PBS (below).
Figure S20. ESR spectra of the complex 2 trapped by TEMP in CH$_3$CN (above) and PBS (below).
**Figure S21.** ESR spectra of the complex 3 trapped by TEMP in CH$_3$CN (above) and PBS (below).
Figure S22. ESR spectra of the complex 4 trapped by TEMP in CH$_3$CN (above) and PBS (below).
Table S2. Singlet oxygen quantum yields in CH$_3$CN and aqueous solution. Average of three independent measurements. n.d. = not detectable.

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<th>direct 450 nm D$_2$O</th>
<th>indirect 450 nm CH$_3$CN</th>
<th>indirect 450 nm PBS</th>
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Figure S23. HPLC chromatogram (Method: M1) of caffeine (internal standard, above) and 1 after 48 h (below) incubation in human pooled plasma.

Figure S24. HPLC chromatogram (Method: M1) of caffeine (internal standard, above) and 2 after 48 h (below) incubation in human pooled plasma.
Figure S25. HPLC chromatogram (Method: M2) of caffeine (internal standard, above) and 3 after 48 h (below) incubation in human pooled plasma.

Figure S26. HPLC chromatogram (Method: M2) of caffeine (internal standard, above) and 4 after 48 h (below) incubation in human pooled plasma.
Figure S27. Temporal change of the UV/Vis spectra of 1 by irradiation at 540 nm in CH$_3$CN.

Figure S28. Temporal change of the UV/Vis spectra of 2 by irradiation at 540 nm in CH$_3$CN.
Figure S29. Temporal change of the UV/Vis spectra of 3 by irradiation at 540 nm in CH$_3$CN.

Figure S30. Temporal change of the UV/Vis spectra of 4 by irradiation at 540 nm in CH$_3$CN.
Figure S31. Temporal change of the UV/Vis spectra of PpIX by irradiation at 540 nm in CH₃CN.

Figure S32. Time dependent cellular uptake of 1 in HeLa cells.
Figure S33. Time dependent cellular uptake of 2 in HeLa cells.

Figure S34. Time dependent cellular uptake of 3 in HeLa cells.
Figure S35. Time dependent cellular uptake of 4 in HeLa cells.

Figure S36. Comparison of the uptake of 1-4 in HeLa cells after 12 h incubation.
Table S3. Distribution coefficients of 1-4 between an organic octanol and aqueous phosphate buffer saline phase.

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<th>Compound</th>
<th>logP</th>
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<tr>
<td>1</td>
<td>+ 0.7 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>+ 0.6 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>+ 1.8 ± 0.3</td>
</tr>
<tr>
<td>4</td>
<td>+ 1.5 ± 0.3</td>
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</table>

Figure S37. Cell uptake mechanism study of 1 (10 μM, 2% DMSO, υ%) in the presence of different inhibitors/conditions. Endocytic inhibition: NH₄Cl (50 mM) or chloroquine (100 μM), metabolic inhibition: 2-Deoxy-D-glucose (50 mM) and oligomycin (5 μM), cation transporter inhibition: Et₄NCl, low temperature: incubation at 4°C, control: incubation at 37°C.
**Figure S38.** Cell uptake mechanism study of 2 (10 μM, 2% DMSO, v%) in the presence of different inhibitors/conditions. Endocytic inhibition: NH₄Cl (50 mM) or chloroquine (100 μM), metabolic inhibition: 2-Deoxy-D-glucose (50 mM) and oligomycin (5 μM), cation transporter inhibition: Et₄NCl, low temperature: incubation at 4°C, control: incubation at 37°C.

**Figure S39.** Cell uptake mechanism study of 3 (10 μM, 2% DMSO, v%) in the presence of different inhibitors/conditions. Endocytic inhibition: NH₄Cl (50 mM) or chloroquine (100 μM), metabolic inhibition: 2-Deoxy-D-glucose (50 mM) and oligomycin (5 μM), cation transporter inhibition: Et₄NCl, low temperature: incubation at 4°C, control: incubation at 37°C.
Figure S40. Cell uptake mechanism study of 4 (10 μM, 2% DMSO, v%) in the presence of different inhibitors/conditions. Endocytic inhibition: NH₄Cl (50 mM) or chloroquine (100 μM), metabolic inhibition: 2-Deoxy-D-glucose (50 mM) and oligomycin (5 μM), cation transporter inhibition: Et₄NCl, low temperature: incubation at 4°C, control: incubation at 37°C.
**Figure S41.** Confocal luminescence image of HeLa cells incubated with the compounds **1-4** (10 μM, 2% DMSO, v%) for 6 h at 37°C in the dark. The investigated Ru complexes were detected using their luminescence (Ru, $\lambda_{\text{ex}} = 458$ nm, $\lambda_{\text{em}} = 600 - 750$ nm) properties.
**Figure S42.** Cellular distribution (Cyto. = Cytoplasm, Mito. = Mitochondria, Lys. = Lysosome, Nuc. = Nucleus) of 1-4 (10 μM, 2% DMSO, v%) in HeLa cells after 6 h incubation in the dark determined by ICP-MS. Error bars: Nuc.: 0.3-0.5%, Lys.: 0.6-2.3%, Mito.: 1.6-3.3%, Cyto.: 6.8%-9.1%, Control: 5.1-8.2%.
**Table S4.** IC$_{50}$ values (in μM) in the dark and upon 1P irradiation at 480 nm (10 min, 3.1 J/cm$^2$) and 540 nm (40 min, 9.5 J/cm$^2$) of 1-4 in comparison to [Ru(phen)$_3$]$^{2+}$, [Ru(bphen)$_3$]$^{2+}$, cisplatin and Protoporphyrin IX (PpIX) in non-cancerous retinal pigment epithelium (RPE-1) and human cervical carcinoma (HeLa). Average of three independent measurements. n.d. = not determinable.

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<td>&gt;100</td>
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<tr>
<td>4</td>
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<td>&gt;100 n.d.</td>
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<td>&gt;100 n.d.</td>
<td>&gt;100 n.d.</td>
<td>&gt;100 n.d.</td>
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<tr>
<td>[Ru(bphen)$_3$]$^{2+}$</td>
<td>12.1 ± 0.6</td>
<td>0.9 ± 0.2</td>
<td>13.4</td>
<td>1.2 ± 0.3</td>
<td>10.1</td>
<td>8.5 ± 0.4</td>
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<tr>
<td>PpIX</td>
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<td>&gt;26.3</td>
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<tr>
<td>cisplatin</td>
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<td>-</td>
<td>10.5 ± 0.8</td>
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**Table S5.** IC$_{50}$ values (in μM) in the dark and upon 1P irradiation at 480 nm (10 min, 3.1 J/cm$^2$) and 540 nm (40 min, 9.5 J/cm$^2$) of 1-4 in comparison to [Ru(phen)$_3$]$^{2+}$, [Ru(bphen)$_3$]$^{2+}$, cisplatin and Protoporphyrin IX (PpIX) in mouse colon carcinoma (CT-26) and human glioblastoma astrocytoma (U373) cells. Average of three independent measurements. n.d. = not determinable.

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<td>&gt;100 n.d.</td>
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<td>&gt;100 n.d.</td>
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<tr>
<td>[Ru(bphen)$_3$]$^{2+}$</td>
<td>2.8 ± 0.5</td>
<td>0.4 ± 0.2</td>
<td>7.0</td>
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<td>cisplatin</td>
<td>2.8 ± 0.4</td>
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<td>8.7 ± 0.6</td>
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Figure S43. Cell death mechanism study of 1-4 with the IC$_{50}$ value of the corresponding compound in HeLa cells upon irradiation at 480 nm (10 min, 3.1 J/cm$^2$) and in the presence of different inhibitors by determination of the cell viability. Autophagy inhibitor: 3-Methyladenine (100 μM), apoptosis inhibitor: Z-VAD-FMK (20 μM), paraptosis inhibitor: Cycloheximide (0.1 μM), necrosis inhibitor: Necrostatin-1 (60 μM).

Figure S44. Apoptosis mechanism study of 1-4 with half of the IC$_{50}$ value of the corresponding compound in HeLa cells upon irradiation at 480 nm (10 min, 3.1 J/cm$^2$) by determination of the caspase 3/7 activity.
Figure S45. One- (OPM, $\lambda_{ex} = 458$ nm, $\lambda_{em} = 600 - 750$ nm) and two-photon (TPM, $\lambda_{ex} = 800$ nm, $\lambda_{em} = 600 - 750$ nm) excited Z-stack images in HeLa MCTS after incubation of 1 after 12 h (10 $\mu$M, 2% DMSO, v%). a) Z-axis images scanning from the top to the bottom of an intact spheroid. b) 3D z-stack of an intact spheroid.

Figure S46. One- (OPM, $\lambda_{ex} = 458$ nm, $\lambda_{em} = 600 - 750$ nm) and two-photon (TPM, $\lambda_{ex} = 800$ nm, $\lambda_{em} = 600 - 750$ nm) excited Z-stack images in HeLa MCTS after incubation of 3 after 12 h (10 $\mu$M, 2% DMSO, v%). a) Z-axis images scanning from the top to the bottom of an intact spheroid. b) 3D z-stack of an intact spheroid.
Figure S47. One- (OPM, $\lambda_{ex} = 458$ nm, $\lambda_{em} = 600 – 750$ nm) and two-photon (TPM, $\lambda_{ex} = 800$ nm, $\lambda_{em} = 600 - 750$ nm) excited Z-stack images in HeLa MCTS after incubation of 4 after 12 h (10 $\mu$M, 2% DMSO, v/v%). a) Z-axis images scanning from the top to the bottom of an intact spheroid. b) 3D z-stack of an intact spheroid.
**Figure S48.** Representative image of the growth inhibition assay in HeLa MCTS. The MCTS were treated with the compounds 1-4 (20 μM, 2% DMSO, v%). MCTS were kept in the dark.
Figure S49. Representative image of the growth inhibition assay in HeLa MCTS. The MCTS were treated with the compounds 1-4 (20 μM, 2% DMSO, v%). The MCTS were exposed to a one-photon irradiation (500 nm, 10 J/cm²) on day 1.
Figure S50. Representative image of the growth inhibition assay in HeLa MCTS. The MCTS were treated with the compounds 1-7 (20 μM, 2% DMSO, v%). The MCTS were exposed to a two-photon irradiation (800 nm, 10 J/cm², section interval of 5 μm) on day 1.
Figure S51. Time dependent biodistribution of 2 in A549 tumour-bearing mice. Ordinate axis: the Ru element content (μg/g).
Figure S52. Representative image of the A549 tumour-bearing mice on day 17.
Figure S53. Picture of tumour H&E stained slices after different treatments, from left to right: NaCl+dark, 2+Dark, NaCl+OP, 2+OP, NaCl+TP, 2+TP. Scale bars: 50 μm.

Figure S54. Picture of organ H&E stained slices after different treatments, from left to right: NaCl+dark, 2+Dark, NaCl+OP, 2+OP, NaCl+TP, 2+TP. Scale bars: 50 μm.
REFERENCES


