Supplementary Information (SI) for Chemical Science.

Toward Photodynamic Detection and Photodynamic Therapy of Tumours Over-Expressing Carbonic Anhydrase IX with a Phosphorescent Organometallic Iridium(III) Antibody Conjugate

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Supporting Information

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12. Live cell confocal microscopy

13. Cytotoxicity and phototoxicity assays

1. Instrumentation

^{1}H and {^{1}H}^{13}C NMR spectra were acquired using a Bruker Ascend 500 MHz instrument fitted with a cryoprobe. ^{1}H NMR spectra were acquired at 500 MHz and {^{1}H}^{13}C NMR spectra were acquired as proton decoupled spectra at 126 MHz and signals were referenced to the residual solvent signal. All NMR spectra were acquired at 25 ºC.

Electrospray ionisation mass spectrometry measurements were acquired on an Exactive Plus Orbitrap Infusion mass spectrometer (Exactive Series, 2.8 Build 268801, ThermoFisher Scientific). Intact protein mass spectra were acquired with an Agilent 6545XT QTOF coupled to an Agilent 1200 Infinity Series LC. Protein desalting and chromatographic separation was performed using an Agilent Poroshell C18 2.1 x 75 mm, 5µm column using 10% (v/v) acetonitrile ported to waste (0 – 3min). Upon desalting of the sample, the flow was ported back into the ESI source for subsequent gradient elution with 10% (v/v) to 70% (v/v) acetonitrile/water with 0.1% formic acid over 8 min at 0.5 mL/min.

ELISA and MTT assay measurements were carried out using a FLUOstar Omega microplate reader and analysed with MARS data analysis software.

UV-Visible spectra were acquired using a Shimadzu UV-1900i spectrophotometer, from 800 nm to 200 nm. Fluorescence spectra were with a Varian Cary Eclipse fluorescence spectrometer. Time-resolved emission measurements were performed using a nanosecond Nd:YAG/optical parametric amplifier laser system (EKSPLA, NT340, 10 Hz) set to 650 nm as the excitation source. Emission was collected with a lens and focussed on to the entrance slit of a spectrometer (Acton SpectraPro 300i) and detected with a photomultiplier tube (R928) wired for rapid time response. The signal was acquired on a LeCroy WaveSurfer digital oscilloscope. Two photon-induced emission spectra were recorded using the 800 nm output from a mode-locked Titanium:sapphire laser (Coherent

Mira/RegA, ~50 fs pulse width) and the emission was acquired with a fibre-optic-based spectrometer (ThorLabs, CCS200) simply held against the cuvette. Samples for excited state lifetime and two-photon induced emission measurements were degassed by successive freeze-pump-thaw cycles.

Confocal fluorescence microscopy images were acquired with a Zeiss Elyra LSM880 using a 40x air objective. Samples were incubated at 37 ºC in a 5% CO_2 atmosphere during imaging.

Cyclic voltammograms were recorded with an Edaq ER466 Integrated Potentiostat System and data processed with EDAQ ECHEM V2.2.3 software

Semi-preparative RP-HPLC purification was carried out using an Agilent 1260 Infinity II Preparative LC System equipped with an Agilent Pursuit XR column (21 mm x 250 mm, 5 µm) and various gradient elutions of acetonitrile in water with 0.1% formic acid at a flow rate of 12 mL/min. Fractions were collected based on absorbance at $\lambda = 254$ nm. Analytical RP-HPLC traces were acquired using an Agilent 1290 LC system equipped with a Phenomenex Luna column (C18, 150 mm x 4.6 mm) and gradient elution of acetonitrile in water with 0.1% trifluoroacetic acid (5-95% acetonitrile in water over 25 minutes).

Protein samples were analysed on an Agilent 1100 Series using a Phenomenex Yarra SEC-3000 3 mm 4.6 × 300 mm column, with a flow rate of 0.35 mL/min and a mobile phase of 0.1 M phosphate buffered saline pH 6.8 with 5% isopropanol.

2. Materials and reagents

All reagents were purchased from standard commercial sources. Azido-dPEG7-amine was purchased from BroadPharm. H-girentuximab was provided by Telix Pharmaceuticals.

3. Synthesis and characterisation

3.1. $[Ir(ppy)_2(pytr\text{-}NH_2)]^+$

2-ethynylpyridine (20 mg, 0.19 mmol) was added to a stirred solution of azido-dPEG7-amine (52 mg, 0.13 mmol), copper sulfate pentahydrate (5 mg, 0.02 mmol) and sodium ascorbate (25 mg, 0.13

mmol) in 1:4 water/tetrahydrofuran (15 mL). The reaction mixture was stirred at room temperature under an atmosphere of dinitrogen for 18 h. after which time the solvents were removed by evaporation under reduced pressure to give a dark brown oil. To this oil was added methanol/dichloromethane (1:4, 15 mL) and $[Ir(ppy)_2(\mu\text{-}Cl)]_2$ (88 mg, 0.082 mmol). The mixture was stirred overnight at room temperature under a dinitrogen atmosphere. The solvents were removed by evaporation under reduced pressure then acetonitrile was added and the mixture filtered through Celite. The filtrate was evaporated to dryness and the resulting tacky yellow solid was purified by semi-preparative reversed phase high performance liquid chromatography. The appropriate fractions were combined and lyophilised to give the product as a tacky yellow solid (27 mg, 19%). ^1H NMR (500 MHz; CD_3CN): δ 3.52 (m, 28H), 3.69 (dd, 2H), 4.59 (t, 2H), 6.26 (ddd, 2H), 6.83 (td, 1H), 6.91 (td, 1H), 6.96 (td, 1H), 7.10-7.02 (m, 4H), 7.36 (ddd, 1H), 7.64 (ddd, 1H), 7.70 (ddd, 1H), 7.74 (dd, 1H), 7.78 (dd, 1H), 7.84-7.82 (m, 2H), 7.86 (d, 2H), 8.08-8.06 (m, 2H), 8.26 (dt, 1H), 9.00 (s, 1H). ^{13}C NMR (126 MHz; CD3CN): δ 40.24, 52.93, 53.07, 67.34, 69.05, 70.08, 70.48, 70.58, 70.60, 70.63, 70.65, 70.72, 70.75, 70.78, 70.85, 70.92, 70.98, 120.58, 120.75, 123.09, 123.58, 123.82, 124.29, 124.50, 125.31, 125.76, 127.66, 127.81, 130.59, 131.26, 132.31, 132.78, 139.42, 139.49, 140.69, 145.18, 145.27, 147.59, 149.45, 150.19, 150.45, 150.58, 151.24, 168.18, 168.61. ESI-MS (positive ion mode, 1:1 MeCN/H_2O and 0.1% formic acid) $[M+H]^{2+}$: 499.6935, calculated for $(C_{45}H_{56}IrN_7O_7)^{2+}$: 499.6930. R_t = 13.2 min.

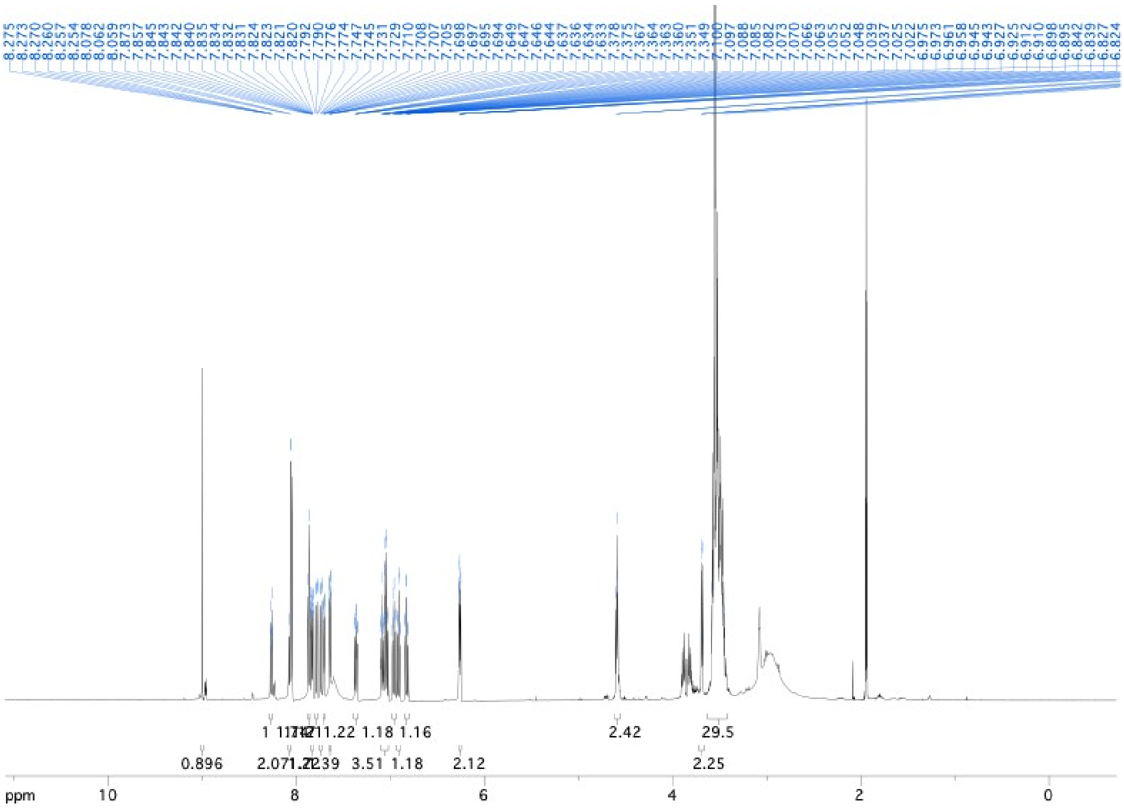

Figure S1: ^{1}H NMR spectrum of $[Ir(ppy)_2(pytr\text{-}NH_2)]^+$ (500 MHz, CD_3CN)

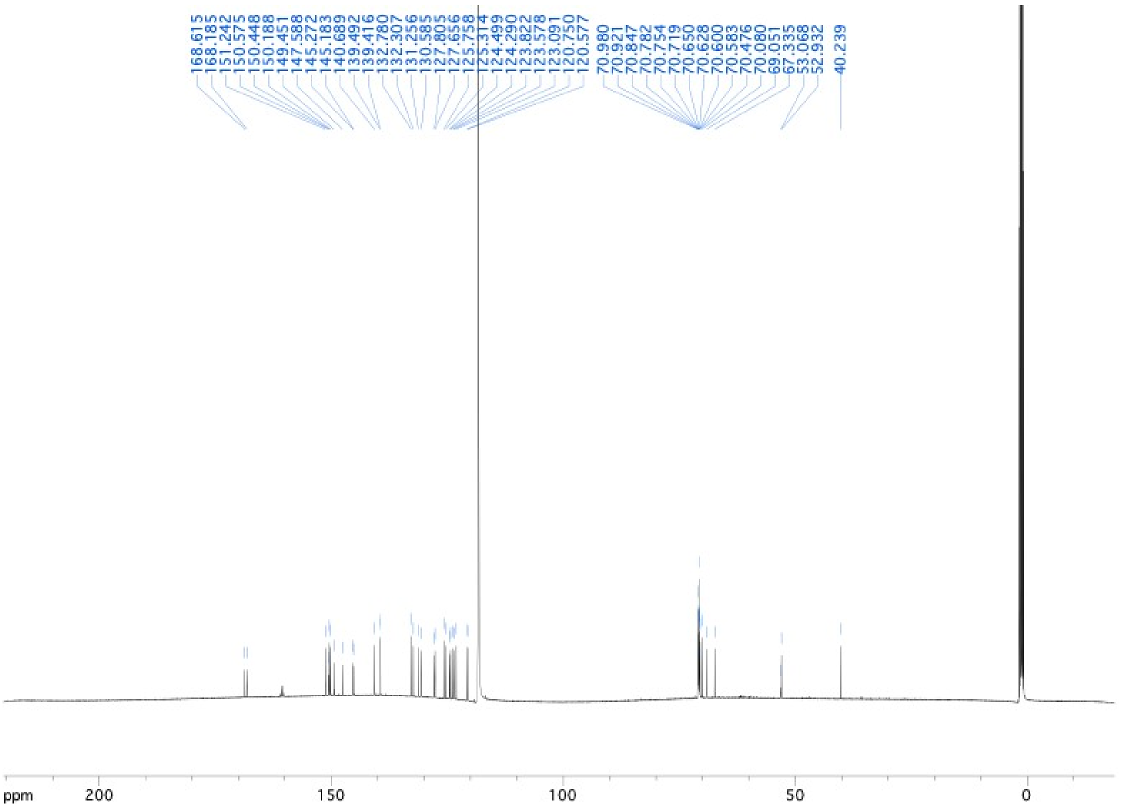

Figure S2: ^{13}C NMR of $[Ir(ppy)_2(pytr\text{-}NH_2)]^+$ (126 MHz, CD_3CN)

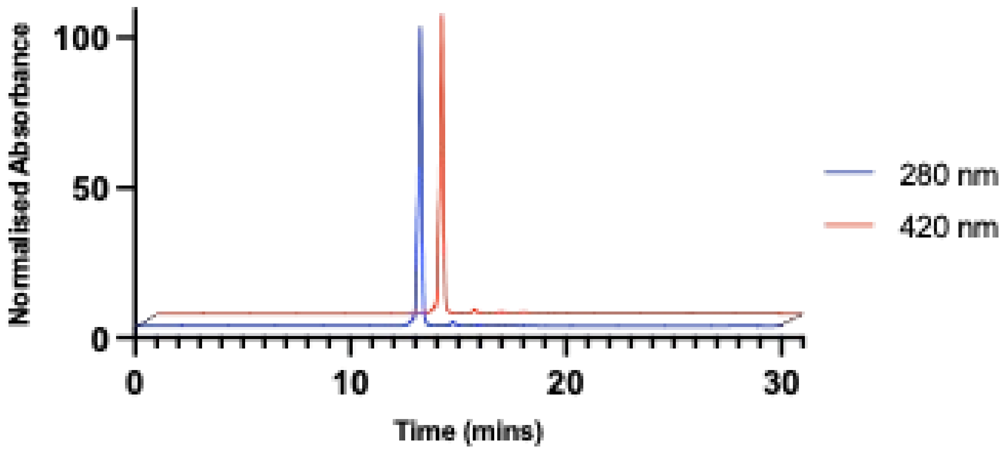

Figure S3: Analytical RP-HPLC of $[Ir(ppy)_2(pytr\text{-}NH_2)]^+$collected at 280 and 420 nm. $R_t = 13.2$ min (> 98% purity).

3.2. $[Ir(piq)_2(pytr\text{-}NH_2)]^+$

2-ethynylpyridine (21 mg, 0.20 mmol) was added to a stirred solution of azido-dPEG7-amine (55 mg, 0.14 mmol), copper sulfate pentahydrate (7 mg, 0.03 mmol) and sodium ascorbate (27 mg, 0.14 mmol) in 1:4 water/tetrahydrofuran (15 mL). The reaction mixture was stirred at room temperature under an atmosphere of dinitrogen for 18 h. after which time the solvents were removed by evaporation under reduced pressure to give a dark brown oil. To this oil was added methanol/dichloromethane (1:4, 15 mL) and $[Ir(piq)_2(\mu\text{-}Cl)]_2$ (108 mg, 0.085 mmol). The mixture was stirred overnight at room temperature under a dinitrogen atmosphere. The solvents were removed by evaporation under reduced pressure then acetonitrile was added and the mixture filtered through Celite. The filtrate was evaporated to dryness and the resulting tacky red solid was purified by semi-preparative reversed phase high performance liquid chromatography. The appropriate fractions were combined and lyophilised to give the product as a tacky yellow solid (33 mg, 20%). ^{1}H NMR (500 MHz; CD_3CN): δ 3.09 (s, 2H), 3.89-3.27 (m, 30H), 4.58 (t, 2H), 6.33 (dd, 1H), 6.42 (dd, 1H), 6.81 (t, 1H), 6.92 (t, 1H), 7.09 (t, 1H), 7.17 (t, 1H), 7.37 (t, 1H), 7.47 (d, 1H), 7.51 (d, 1H), 7.58 (d, 1H), 7.66 (t, 1H), 7.76 (dt, 1H), 7.87 (dt, 3H), 8.09-8.02 (m, 3H), 8.27 (d, 1H), 8.36 (d, 1H), 8.41 (d, 1H), 8.96 (s, 1H), 9.06-9.04 (m, 2H). ^{13}C NMR (126 MHz; CD_3CN): δ 40.20, 52.91, 67.23, 69.05, 69.94, 70.43, 70.50, 70.54, 70.59, 70.62, 70.73, 70.77, 70.86, 70.89, 70.93, 70.97, 71.03, 122.59, 122.86, 123.32, 123.81, 127.02, 127.12, 127.63, 127.67, 127.75, 128.49, 128.52, 129.87, 129.93, 130.63, 131.21, 131.37, 131.72, 132.84, 132.87, 132.94, 133.30, 138.03, 138.07, 140.72, 141.84, 142.13, 146.65, 146.75, 149.29, 150.25, 150.30, 151.01, 151.29, 154.03, 169.25, 169.56. ESI-MS (positive ion mode, 1:1 MeCN/H_2O and 0.1% formic acid) $[M+H]^{2+}$: 549.7093, calculated for $(C_{53}H_{60}IrN_7O_7)^{2+}$: 549.7089. R_t = 15.9 min.

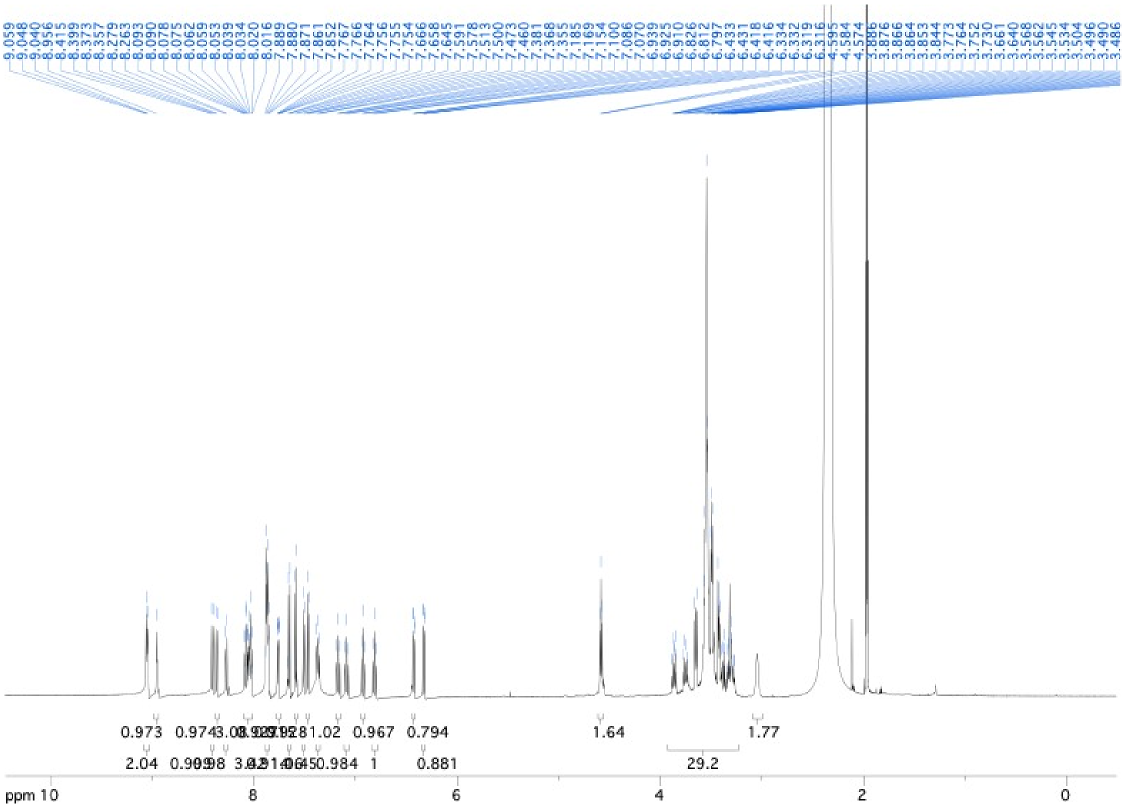

Figure S4: ^1H NMR spectrum of $[Ir(piq)_2(pytr\text{-}NH_2)]^+$ (500 MHz, CD_3CN)

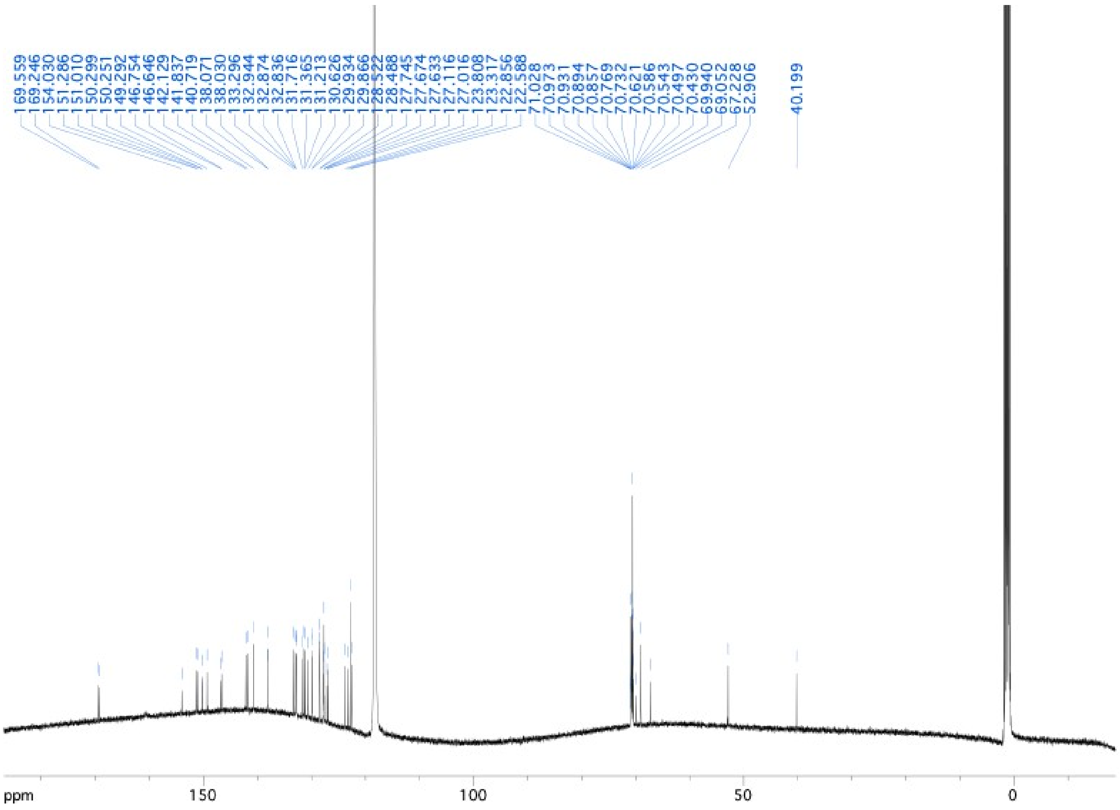

Figure S5: ^{13}C NMR of $[Ir(piq)_2(pytr\text{-}NH_2)]^+$ (126 MHz, CD_3CN)

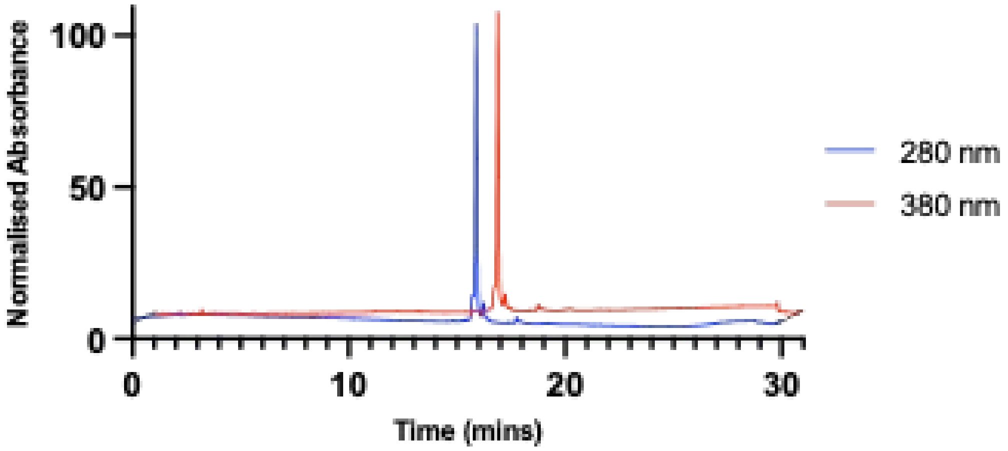

Figure S6: Analytical RP-HPLC of $[Ir(piq)_2(pytr\text{-}NH_2)]^+$ collected at 280 and 380 nm. $R_t = 15.9$ min (> 88% purity).

3.3. $[Ir(ppy)_2(pytr\text{-}SqOEt)]^+$

A solution of $[Ir(ppy)_2(pytr\text{-}NH_2)]$ (50 mg, 0.045 mmol), diethyl squarate (22 mg, 0.13 mmol) and N,N-diisopropylethylamine (100 µL) was heated to 120 ºC with microwave irradiation for 20 min. The solvents were removed by evaporation under reduced pressure and the residue purified by reverse-phase high performance liquid chromatography. The appropriate fractions were combined and lyophilised to give the product as a tacky yellow solid (34 mg, 61%). ^{1}H NMR (500 MHz; CD_3CN): δ 1.37 (m, 3H), 3.92-3.39 (m, 30H), 4.59 (t, 2H), 4.62 (s, 2H), 6.26 (ddd, 2H), 6.83 (td, 1H), 6.91 (td, 1H), 6.96 (td, 1H), 7.05 (tdd, 2H), 7.09 (ddd, 1H), 7.36 (ddd 1H), 7.64 (ddd, 1H), 7.71 (dt, 1H), 7.74 (dd, 1H), 7.78 (dd, 1H), 7.82 (dt, 1H), 7.86 (td, *J* = 2H), 8.08-8.04 (m, 3H), 8.29 (d, 1H), 8.60 (s, 1H), 9.12 (s, 1H). {^{1}H}^{13}C NMR (126 MHz; CD3CN) *δ* 16.2, 30.9, 53.1, 69.1, 70.0, 71.0, 71.0, 71.0, 71.1, 71.1, 71.1, 71.1, 71.1, 118.3, 120.6, 120.8, 123.1, 123.6, 124.0, 124.4, 124.6, 125.4, 125.8, 127.7, 128.2, 130.6, 131.3, 132.4, 132.8, 139.5, 139.5, 140.8, 145.3, 145.3, 147.7, 149.4, 150.3, 150.6, 150.7, 151.2, 167.1, 168.2, 168.7. ESI-MS (positive ion mode, 1:1 MeCN/H_2O and 0.1% formic acid) $[M+H]^{2+}$: 561.7041, calculated for $(C_{51}H_{60}IrN_7O_{10})^{2+}$: 561.7010; $[M]^+$: 1122.3967, calculated for $(C_{51}H_{59}IrN_7O_{10})^+$: 1122.3947. R_t = 15.7 min.

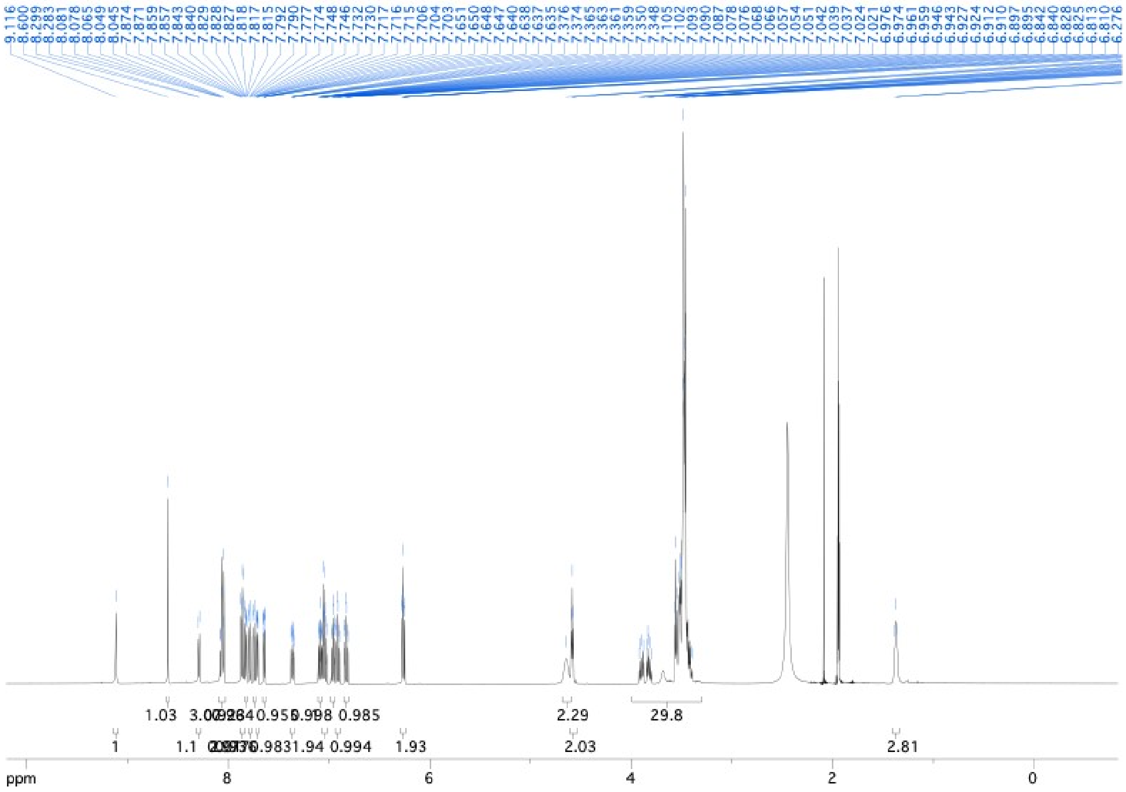

Figure S7: ^{1}H NMR spectrum of $[Ir(ppy)_2(pytr\text{-}SqOEt)]^+$ (500 MHz, CD_3CN)

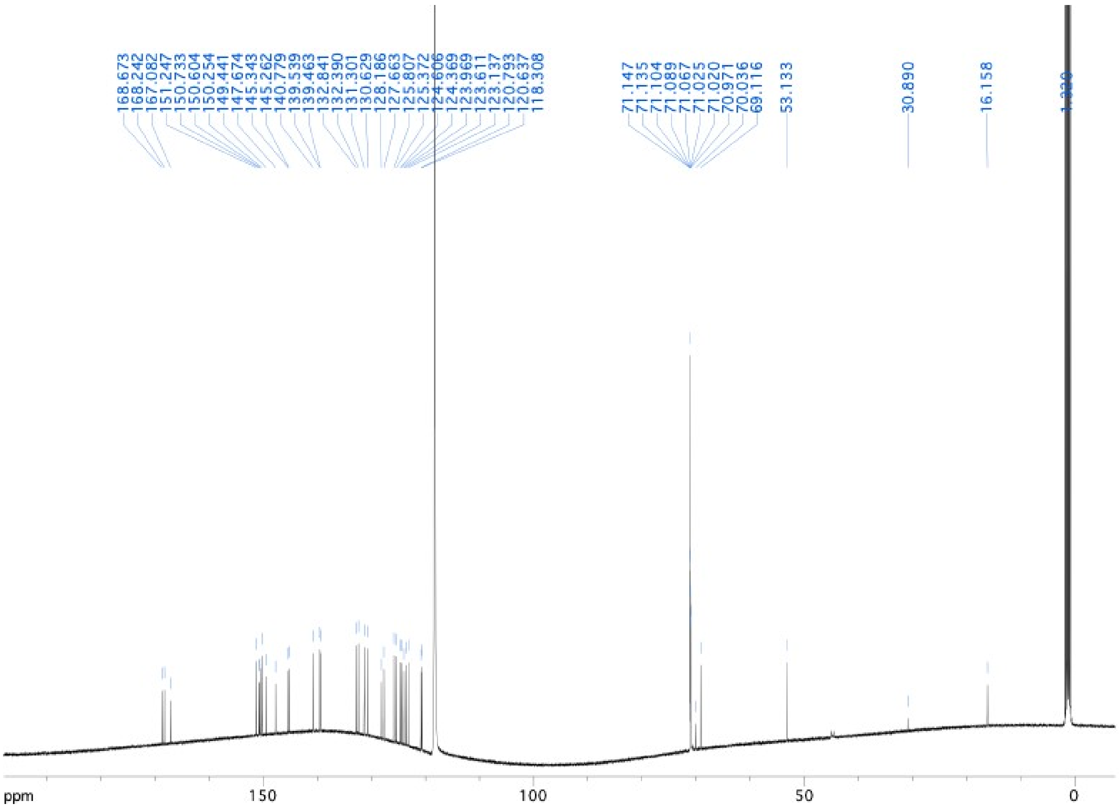

Figure S8: ^{13}C NMR spectrum of [Ir(ppy)$_2$(pytr-SqOEt)]$^+$ (126 MHz, CD$_3$CN)

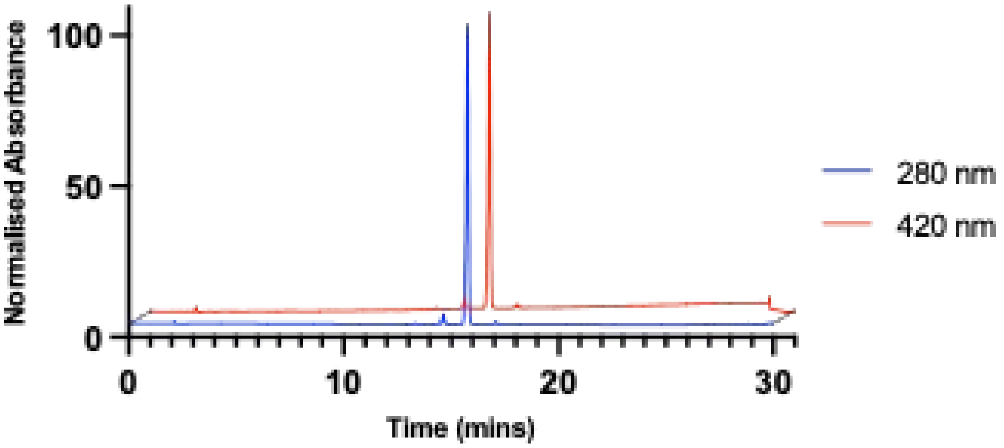

Figure S9: Analytical RP-HPLC of [Ir(ppy)$_2$(pytr-SqOEt)]$^+$ collected at 280 and 420 nm. R$_t$ = 15.7 min (> 98% purity).

3.4. [Ir(piq)$_2$(pytr-SqOEt)]$^+$

A solution of [Ir(piq)$_2$(pytr-NH$_2$)] (30 mg, 0.022 mmol), diethyl squarate (12 mg, 0.068 mmol) and N,N-diisopropylethylamine (100 µL) in ethanol (10 mL) was heated to 120 ºC with microwave irradiation for 20 min. The solvents were removed by evaporation under reduced pressure and the residue purified by reverse-phase high performance liquid chromatography. Purified fractions were lyophilised to give the product as a tacky red solid (17 mg, 58%). ^{1}H NMR (500 MHz; CD_3CN): δ 1.36 (t, 3H), 3.53-3.23 (m, 27H), 3.87-3.70 (m, 3H), 4.54 (t, 2H), 4.63 (s, 2H), 6.30 (dd, 1H), 6.41 (dd, *J* = 7.6, 1H), 6.78 (td, 1H), 6.90 (td, 1H), 7.06 (ddd, 1H), 7.16-7.13 (m, 1H), 7.34 (ddd, 1H), 7.44 (d, 1H), 7.48 (d, 1H), 7.56 (d, 1H), 7.64 (d, 1H), 7.73 (d, 1H), 7.85-7.82 (m, 4H), 8.06-7.99 (m, 3H), 8.25 (d, 1H), 8.34 (d, 1H), 8.38 (d, 1H), 8.63 (s, 1H), 8.98 (s, 1H), 9.02 (t, 2H). {^{1}H}^{13}C NMR (126 MHz; CD_3CN) δ 16.1, 53.2, 69.1, 70.1, 70.9, 71.0, 71.0, 71.0, 71.1, 71.1, 71.1, 71.1, 71.1, 122.7, 122.9, 122.9, 123.3, 123.9, 127.1, 127.2, 127.7, 127.7, 128.1, 128.5, 128.6, 129.9, 130.0, 130.6, 131.3, 131.4, 131.7, 132.8, 132.9, 133.0, 133.3, 138.1, 138.1, 140.8, 141.9, 142.2, 146.7, 146.8, 149.2, 150.4, 151.0, 151.3, 154.2, 167.1, 169.3, 169.6. ESI-MS (positive ion mode, 1:1 MeCN/H_2O and 0.1% formic acid) $[M+H]^{2+}$: 611.7176, calculated for $(C_{59}H_{64}IrN_7O_{10}{}^{2+}$: 611.7167; $[M]^+$: 1222.4299, calculated for $(C_{59}H_{63}IrN_7O_{10})^+$:1222.4261. R_t = 18.6 min.

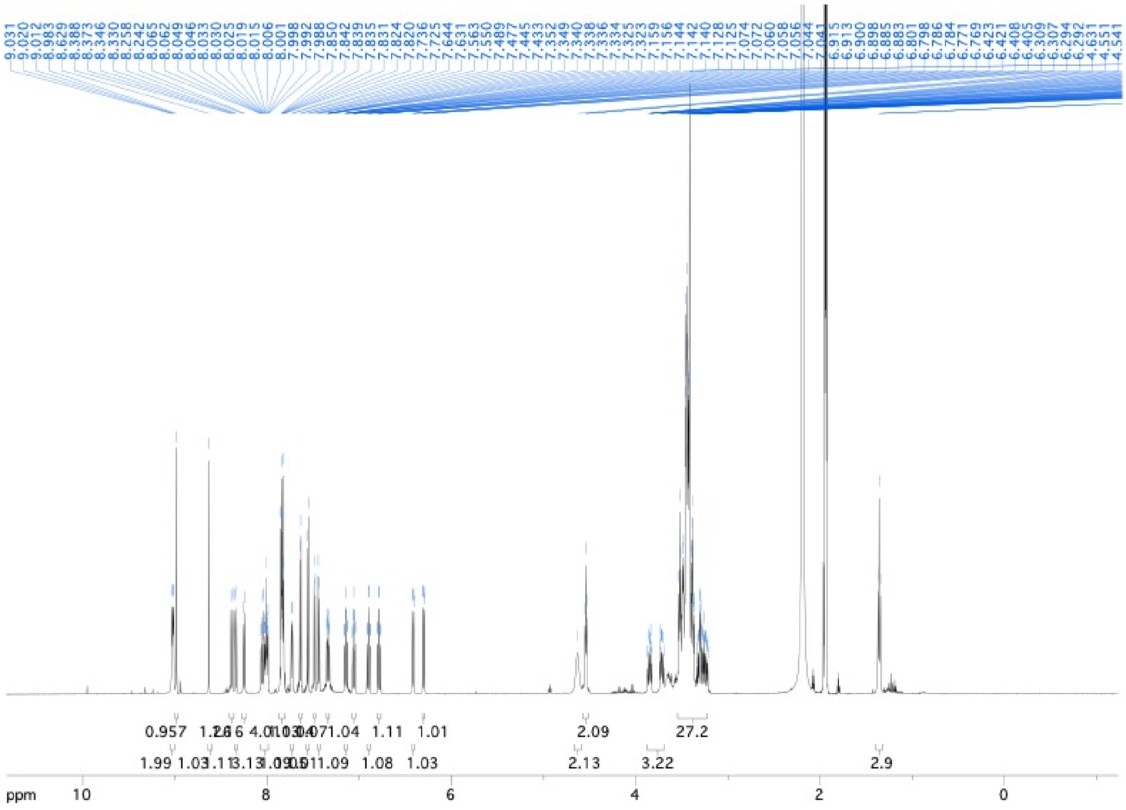

Figure S10: ^{1}H NMR spectrum of $[Ir(piq)_2(pytr\text{-}SqOEt)]^+$ (500 MHz, CD_3CN)

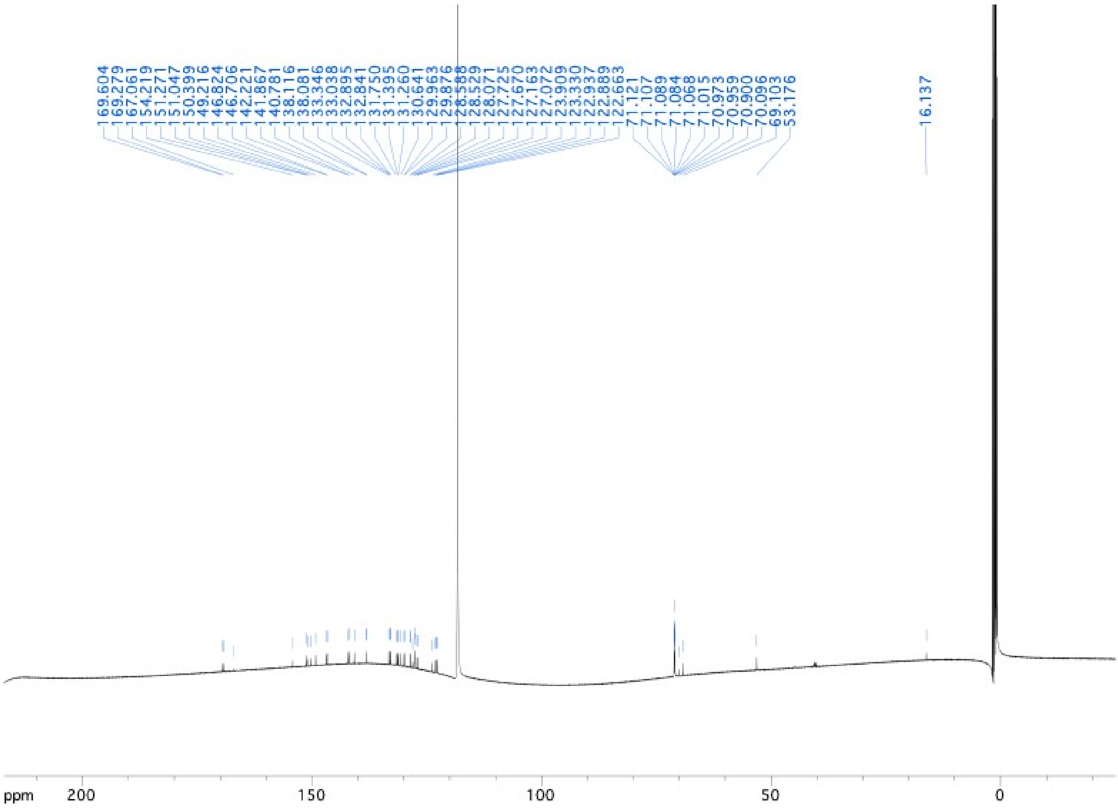

Figure S11: ^{13}C NMR spectrum of [Ir(piq)$_2$(pytr-SqOEt)]$^+$ (126 MHz, CD$_3$CN)

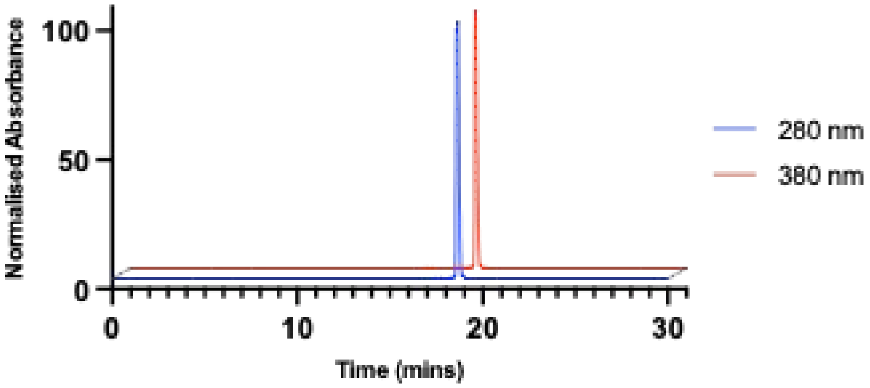

Figure S12: Analytical RP-HPLC of [Ir(piq)$_2$(pytr-SqOEt)]$^+$ collected at 280 and 380 nm. R$_t$ = 18.6 min (> 99% purity).

3.5. [Ir(ppy)$_2$(pytr-Sq-girentuximab)], [Ir(piq)$_2$(pytr-Sq-girentuximab)]

A solution of h-girentuximab (1 mg, 1 mg/mL) and either [Ir(ppy)$_2$(pytr-SqOEt)] or [Ir(piq)$_2$(pytr-SqOEt)] (20 eq) in 0.1 M borate buffer (pH 9) and 5% DMSO was incubated for 18 h at room temperature. The mixture was made up to 2.5 mL with 0.9% saline and purified by size exclusion chromatography (PD-10 Sephadex G25 M with 0.9% saline). Final protein concentration was determined by bicinchoninic acid (BCA) assay. Conjugates were analysed by size exclusion HPLC. [Ir(ppy)$_2$(pytr-Sq-girentuximab)] retention time = 9.8 min, [Ir(piq)$_2$(pytr-Sq-girentuximab)] retention time = 9.4 min, h-girentuximab retention time = 9.5 min.

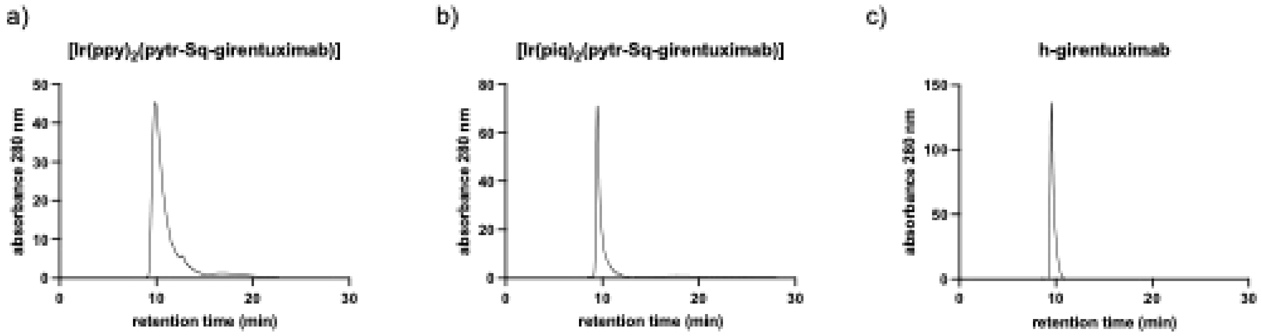

Figure S13: size exclusion chromatograms of [Ir(ppy)$_2$(pytr-Sq-girentuximab)] (a), [Ir(piq)$_2$(pytr-Sq-girentuximab)] (b), and h-girentuximab (c).

4. Cyclic voltammetry

Solutions were made up of analyte (1 mM) and supporting electrolyte (tetrabutylammonium hexafluorophosphate, 0.1 M) in acetonitrile. Solutions were sparged with argon for 5 minutes prior to analysis. Measurements were carried out under an argon atmosphere using a glassy carbon working electrode, Ag^+/AgCl reference electrode and a platinum wire counter electrode. The working electrode was polished with 0.3 mm alumina and water on felt pad, then rinsed with acetonitrile and dried before each measurement. Reported potentials are referenced to ferrocene/ferrocenium.

5. Electronic spectroscopy

Samples for UV-visible spectroscopy were measured at two concentrations (10 μM and 100 μM in acetonitrile). Extinction coefficients were calculated using the Beer-Lambert law. Solutions for fluorescence emission spectroscopy were prepared in acetonitrile to have an absorbance of 0.1 – 0.12 at the excitation wavelength (λ = 380 nm for ppy complexes, λ = 435 nm for piq complexes). Excitation and emission slit widths were 10 nm. For time resolved emission spectroscopy, solutions were prepared in acetonitrile to have an absorbance of 0.1 – 0.12 at the MLCT absorbance maximum and then deoxygenated in degassing cuvettes using successive freeze-pump-thaw cycles.

6. ELISA

Human carbonic anhydrase IX (50 μg) was reconstituted in MilliQ (250 μL) to give a 200 μg/mL stock solution. An aliquot of this stock solution was taken and diluted to 3 μg/mL. 100 μL/well of this solution was used to coat a 96 well plate by incubating overnight at 4 ºC. The plate was then washed with phosphate buffered saline with 0.1% Tween-20 (PBST, 4 x 300 μL/well) and incubated with 1% w/v bovine serum albumin in phosphate buffered saline (PBS, 100 μL/well) for 60 mins at room temperature. Following washing (4 x 300 μL PBST/well), either h-girentuximab, [Ir(ppy)$_2$(pytr-sq-girentuximab)] or [Ir(piq)$_2$(pytr-sq-girentuximab)] were added to appropriate wells, after having been diluted to a range of concentrations spanning 2.28 – 5000 ng/mL from 100 μg/mL stocks (diluent 1% BSA, 0.05% Tween-20 in PBS). The plate was incubated at room temperature for 75 minutes before being washed (PBST, 4 x 300 μL/well). The secondary antibody-horseradish peroxidase conjugate was diluted to 15 ng/mL with 1% BSA, 0.05% Tween-20 in DPBS and 100 μL added to each well. After incubation at room temperature for 75 minutes, 3,3',5,5'-tetramethylbenzidine substrate was added (100 μL/well) and the plate incubated 15 minutes at room temperature. After this time, 2 M H_2SO_4 was added (100 μL/well) and the absorbance of each well was measured at λ = 450 nm.

7. Investigation into the Stability of [Ir(ppy)$_2$(pytr-Sq-girentuximab)] and [Ir(piq)$_2$(pytr-Sq-girentuximab)]

Analysis of samples of [Ir(ppy)$_2$(pytr-Sq-girentuximab)] or [Ir(piq)$_2$(pytr-Sq-girentuximab)] by size-exclusion chromatography (Figure S9) confirmed the conjugates were stable for at least 8 months when stored in 0.9% saline in the fridge (4 °C).

Stability of [Ir(ppy)$_2$(pytr-Sq-girentuximab)] or [Ir(piq)$_2$(pytr-Sq-girentuximab)] when challenged with cysteine and histidine: Solutions of [Ir(ppy)$_2$(pytr-Sq-girentuximab)] or [Ir(piq)$_2$(pytr-Sq-girentuximab)] (4 mg/mL in 0.9% saline) were incubated at 20 ºC for 4 h with 5 mM L-cysteine and 5 mM L-histidine. The fluorescence emission spectrum was measured periodically to assess stability. For [Ir(piq)$_2$(pytr-girentuximab)], λ_{exc} = 435 nm and for [Ir(ppy)$_2$(pytr-girentuximab)] λ_{exc} = 380 nm.

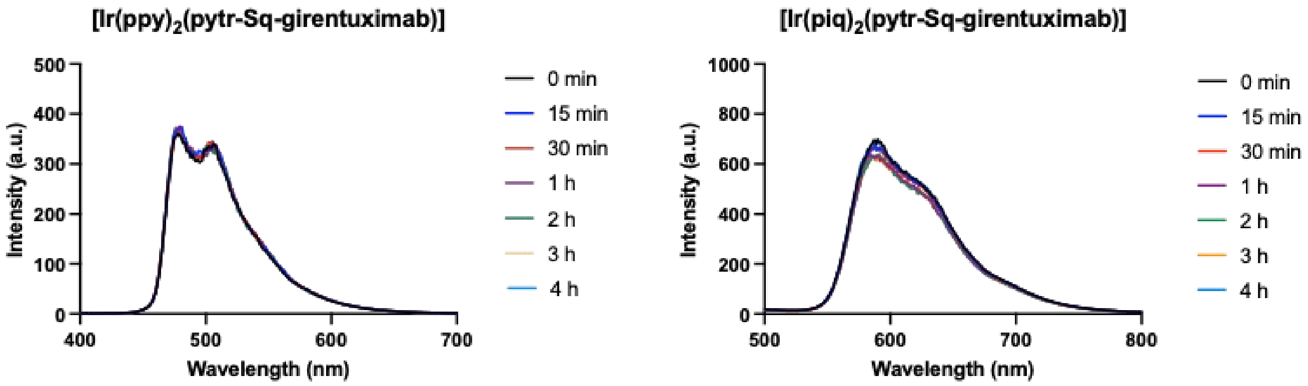

Figure S14: Fluorescence emission spectra for [Ir(ppy)$_2$(pytr-Sq-girentuximab)] (left) or [Ir(piq)$_2$(pytr-Sq-girentuximab)] (right) after incubation with 5 mM L-cysteine and 5 mM L-histidine.

8. Quantum yield of singlet oxygen generation (Φ_Δ) determination

Air equilibrated solutions of [Ir(ppy)$_2$(pytr-NH$_2$)]$^+$, [Ir(piq)$_2$(pytr-NH$_2$)]$^+$ or [Ru(bpy)$_3$]Cl$_2$ (Φ_Δ = 0.57 in air equilibrated acetonitrile) in acetonitrile were adjusted in concentration such that their absorbance at λ = 365 nm was between 0.1 and 0.2. 1,3-diphenylisobenzofuran was added to each solution (10 mM final). Solutions were irradiated at λ = 365 nm and the absorbance at 411 nm monitored. The decrease in absorbance was plotted against irradiation time and the quantum yields of singlet oxygen generation determined using Equation 1. The photooxidation of DPBF was also measured and accounted for in the determination.

$$\Phi_{\Delta,\ sample} = \frac{M_{sample}}{M_{ref}} \times \frac{F_{ref}}{F_{sample}} \times \Phi_{\Delta\ ,ref}$$

Equation 1: Φ_Δ is the quantum yield of singlet oxygen generation, M is the corrected slope of the linear fit of change in absorbance at 411 nm vs irradiation time, $F = 1 – 10^{AL}$ (A = absorbance at λ = 365 nm and L = path length of cell in cm).

The corrected slopes are found by subtracting the slope of the linear fit of the change in absorbance at 411 nm vs irradiation time of the DPBF only from the slope of the linear fit of either the sample or the reference with 10 mM DPBF.

9. Investigation of hydroxyl radical generation using hydroxyphenylfluorescein

Solutions of either [Ir(ppy)$_2$(pytr-NH$_2$)]$^+$ (1.6 µM) or [Ir(piq)$_2$(pytr-NH$_2$)]$^+$ (5 µM) in phosphate buffered saline (PBS, pH 7.4) with hydroxyphenylfluorescein (HPF) (5 µM) were irradiated (λ_{exc} = 420 nm, 26.84 mW/cm^2) for the time intervals indicated and the fluorescence emission spectra measured before and after irradiation. Increase in fluorescence intensity indicates the generation of hydroxyl radical.

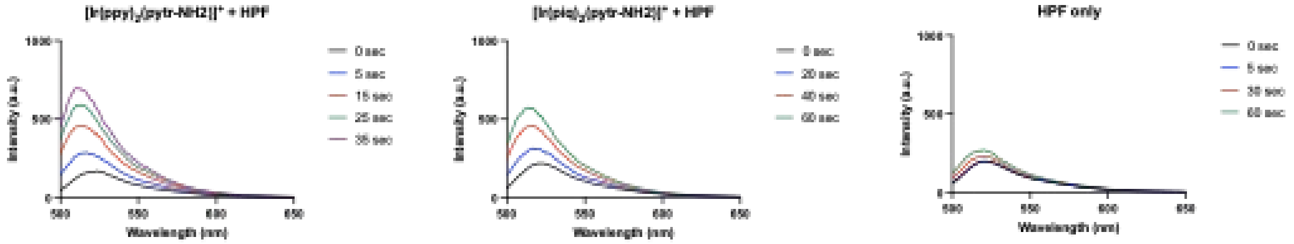

Figure S15: Fluorescence emission spectra of HPF (5 µM) in the presence of [Ir(ppy)$_2$(pytr-NH$_2$)]$^+$ (left), [Ir(piq)$_2$(pytr-NH$_2$)]$^+$ (middle) or PBS (right) after irradiation.

10. Investigation of superoxide anion radical generation using dihydrorhodamine 123

Solutions of either [Ir(ppy)$_2$(pytr-NH$_2$)]$^+$ (0.2 µM) or [Ir(piq)$_2$(pytr-NH$_2$)]$^+$ (1.7 µM) in phosphate buffered saline (pH 7.4) with dihydrorhodamine 123 (DHR) (1.25 µM) were irradiated (λ_{exc} = 420 nm, 26.84 mW/cm^2) for the time intervals indicated and the fluorescence emission spectra measured before and after irradiation. Increase in fluorescence intensity indicates the generation of superoxide anion radical.

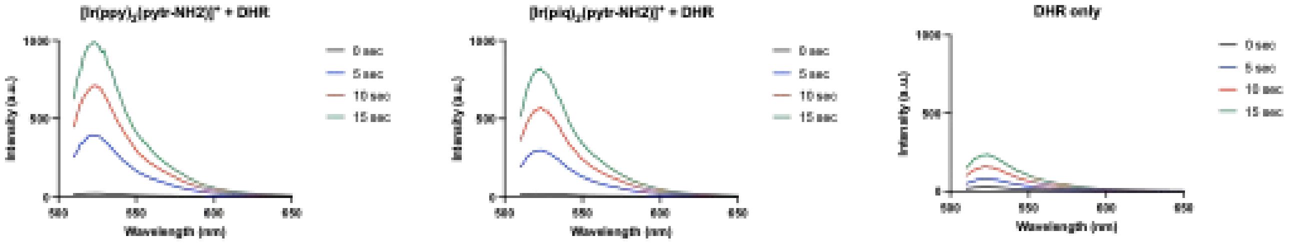

Figure S16: Fluorescence emission spectra for DHR (1.25 µM) in the presence of $[Ir(ppy)_2(pytr-NH_2)]^+$ (left), $[Ir(piq)_2(pytr-NH_2)]^+$ (middle) or PBS (right).

11. Cell culture

Cells were cultured in either McCoy's 5A media (HT-29 cells) supplemented with 10% fetal bovine serum, at 37 ºC in a 5% CO_2 atmosphere. Cells were passaged and all cellular experiments carried out according to standard aseptic procedures.

12. Live cell confocal microscopy

Cells in growth medium were seeded (100 000 cells/well) into poly-L-lysine treated wells of 8-well chamber slides (ibidi) and incubated at 37 ºC in a 5% CO_2 atmosphere for 18 – 24 h to adhere. Cells were then incubated with either $[Ir(piq)_2(pytr\text{-}girentuximab)]$ (1 mg/mL) or $[Ir(piq)_2(pytr-NH_2)]^+$ (10 µM) in complete growth medium. Cells were washed twice with DPBS then media changed into Fluorobrite Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and Hoescht 33342 (20 µg/mL) before imaging with a Zeiss Elyra LSM880 with a 40x air objective lens. Excitation wavelengths were either $\lambda = 458$ nm or $\lambda = 488$ nm as well as $\lambda = 405$ nm. During imaging cells were incubated at 37 ºC in a 5% CO_2 atmosphere.

13. Cytotoxicity and photocytotoxicity assays

Cell cytotoxicity and photocytotoxicity assays were carried out in black-walled, UV-clear flat bottomed 96-well plates that had been poly-L-lysine treated. HT-29 cells were seeded into the inner wells (25 000 cells/well) in complete growth medium, leaving one column without cells. Peripheral wells had 200 µL DPBS/well throughout the entire assay to minimise evaporation from inner wells. Cells were allowed to adhere overnight at 37 ºC in a 5% CO_2 atmosphere. Media was removed and cells were incubated in McCoy's 5A medium supplemented with 1% fetal bovine serum for 1 h at 37 ºC in a 5% CO_2 atmosphere. $[Ir(ppy)_2(pytr\text{-}girentuximab)]$, $[Ir(piq)_2(pytr\text{-}girentuximab)]$ or $[Ir(piq)_2(pytr\text{-}NH_2)]^+$ were serially diluted in complete growth medium to span a concentration range of 10 000 µg/mL – 1 µg/mL (for the girentuximab conjugates) or 10 µM – 1.4 pM (for the pytr-NH_2 complexes) and added to the appropriate wells, with n = 3 for each concentration. Controls were cells treated with complete growth medium only (100% viability) and wells with no cells treated with complete growth medium only (0% viability). Cells were incubated for 1 h at 37 ºC in a 5% CO_2 atmosphere before each well was washed with 3 x 100 µL DPBS. Cells were either kept in the dark at 37 ºC in a 5% CO_2 atmosphere or irradiated (λ_{exc} = 420 nm, 30 minutes, 26.84 mW/cm^2). If cells were to be irradiated, they were media changed into Fluorobrite Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum after washing and before irradiation. Cells were then media changed into fresh McCoy's 5A medium supplemented with 10% fetal bovine serum and incubated overnight at 37 ºC in a 5% CO_2 atmosphere. 10 µL of MTT solution (5 mg/mL in DPBS) was added to each well and the plate incubated for 2 h at 37 ºC in a 5% CO_2 atmosphere. 100 µL solubilising solution (0.01% HCl, 10% Triton X-100, 10 % DMSO in isopropanol) was added to each well and the plate incubated overnight at 37 ºC. Wells were thoroughly mixed and 90 µL from each well transferred into a new UV-clear 96-well plate. The absorbance of each well at λ = 570 nm and λ = 690 nm was measured.

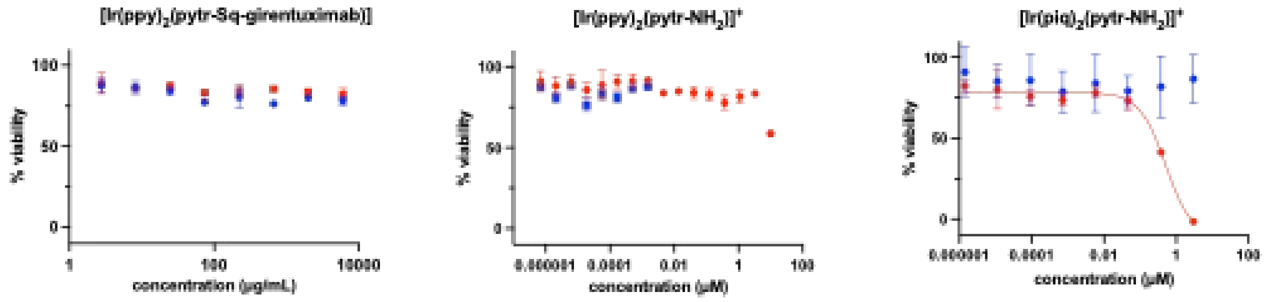

Figure S17: Cellular viability dose-response curves for [Ir(ppy)$_2$(Sq-girentuximab)] (left), [Ir(ppy)$_2$(pytr-NH$_2$)]$^+$ (middle) and [Ir(piq)$_2$(pytr-NH$_2$)]$^+$ (right) in HT-29 cells either in the dark (blue) or with irradiation (red, λ_{ex} = 420 nm, 26.84 mW/cm^2, 30 mins).

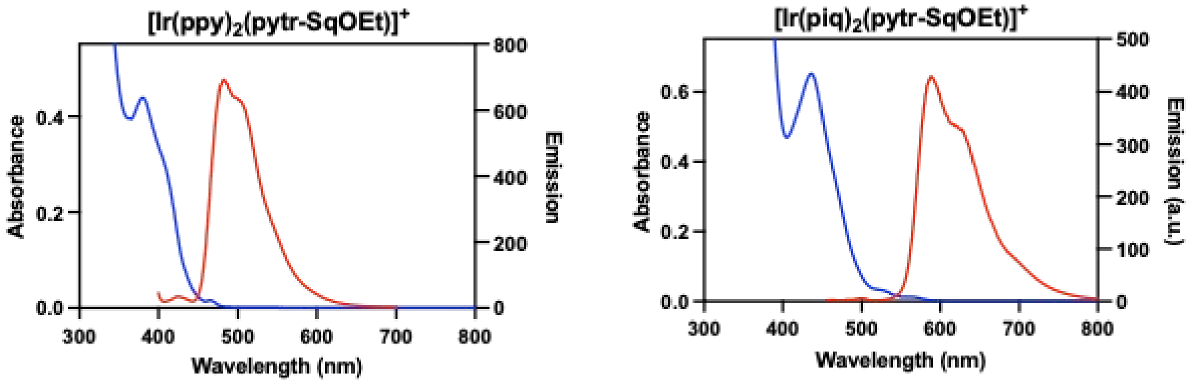

Figure S18: MLCT absorbance (blue) and emission (red) spectra of [Ir(ppy)$_2$(pytr-SqOEt)]$^+$ (left, λ_{ex} = 380 nm) and [Ir(piq)$_2$(pytr-SqOEt)]$^+$ (right, λ_{ex} = 435 nm)

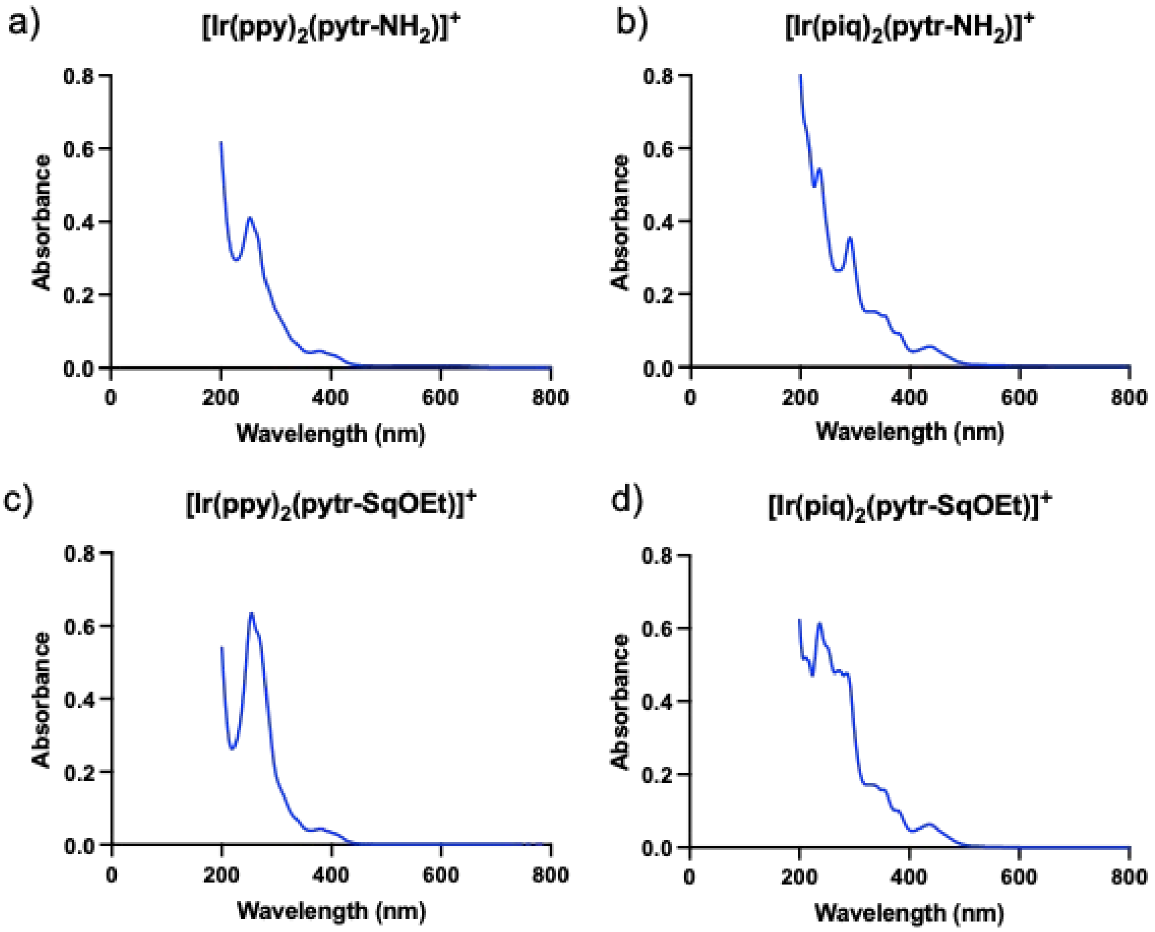

Figure S19: Absorbance spectra of $[Ir(ppy)_2(pytr\text{-}NH_2)]^+$ (a), $[Ir(piq)_2(pytr\text{-}NH_2)]^+$ (b), $[Ir(ppy)_2(pytr\text{-}SqOEt)^+]$ (c), and $[Ir(piq)_2(pytr\text{-}SqOEt)]^+$ (d), 10 µM in acetonitrile.

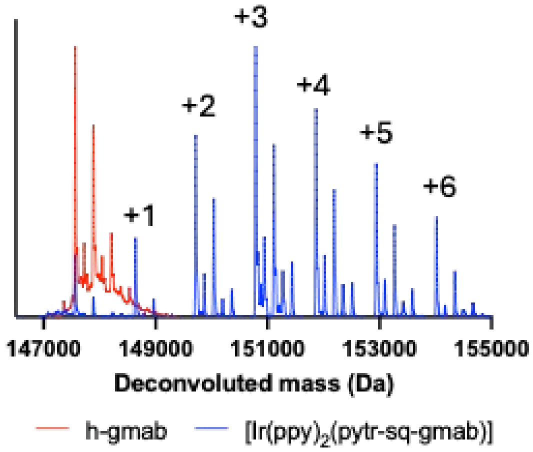

Figure S20: deconvoluted ESI-TOF mass spectra of h-girentuximab (red) and $[Ir(ppy)_2(pytr\text{-}sq\text{-}girentuximab)]$ (blue), showing between zero and six additions of $[Ir(ppy)_2(pytr\text{-}SqOEt)]$.

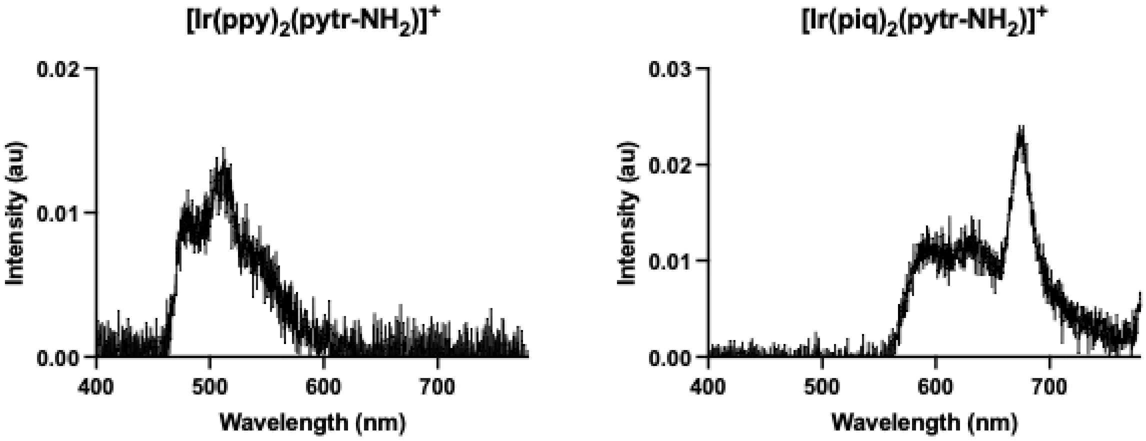

Figure S21: two photon induced emission spectra for [Ir(ppy)$_2$(pytr-NH$_2$)]$^+$ (left) and [Ir(piq)$_2$(pytr-NH$_2$)]$^+$ (right), λ_{ex} = 800 nm.