Supplemental information for:

A Photoactivated Ir(III) Complex Targets Cancer Stem Cells and Induces Secretion of Damage-associated Molecular Patterns in Melamoma Cells Characteristic of Immunogenic Cell Death

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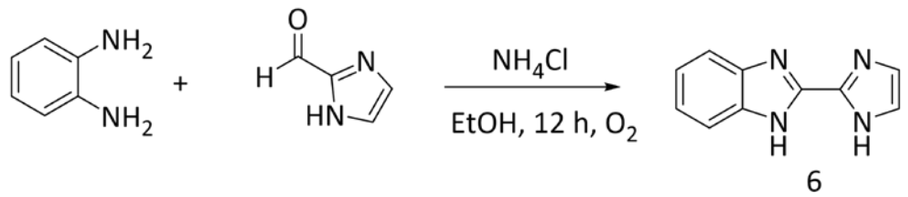
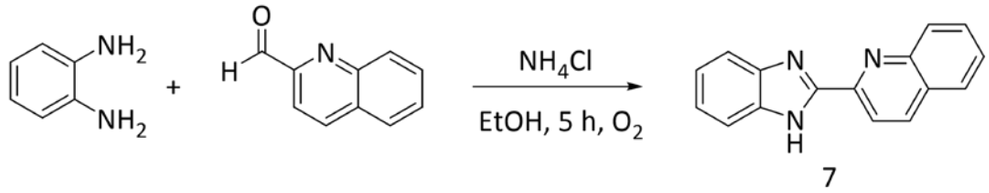


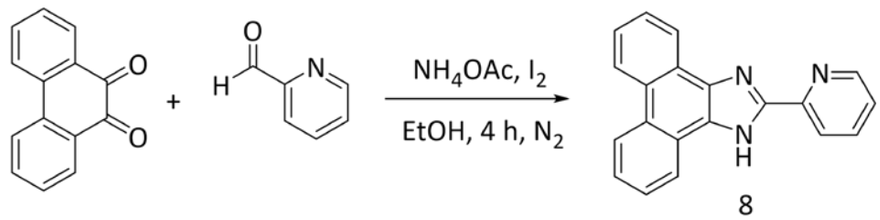
6

Scheme S1. Synthesis of **6**.



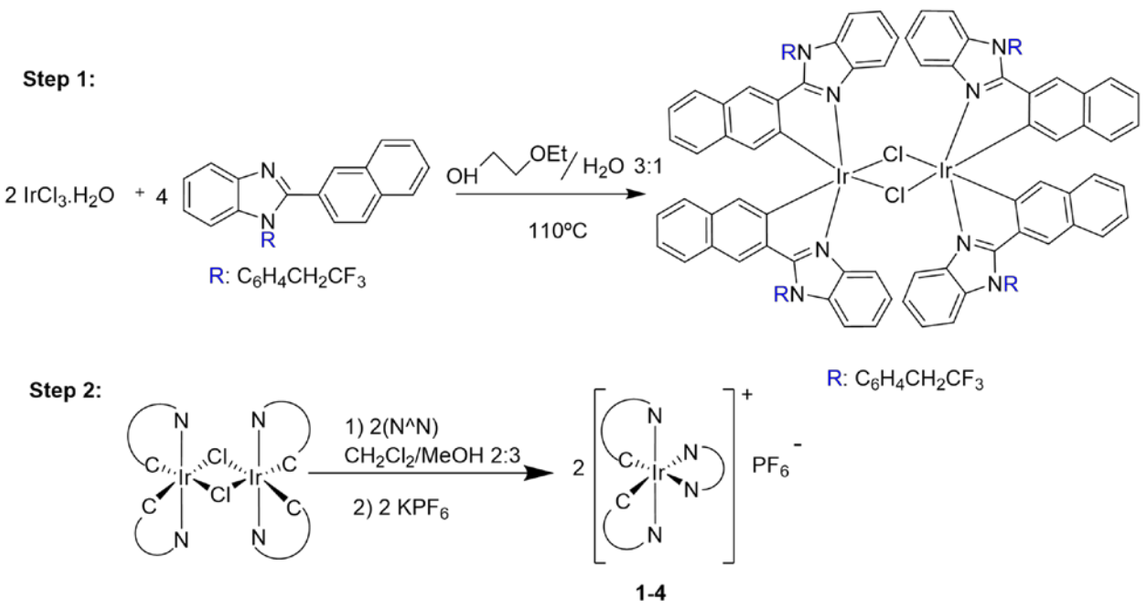
7

Scheme S2. Synthesis of **7**.



8

Scheme S3. Synthesis of **8**.



1-4

Scheme S4. Route of synthesis of the new Ir(III) complexes.

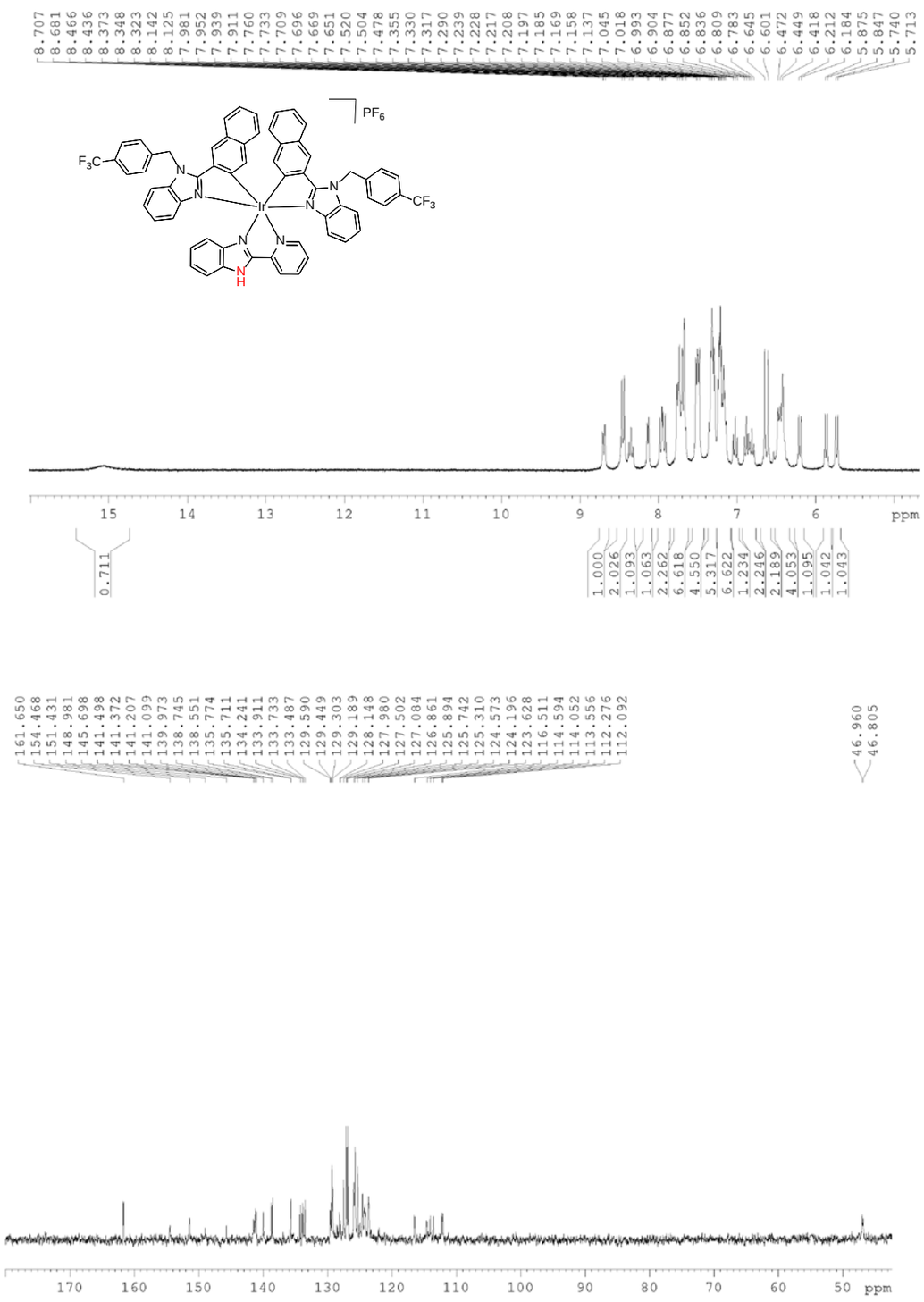


Figure S1. ^{1}H and ^{13}C NMR in [D_6]DMSO for **1**.

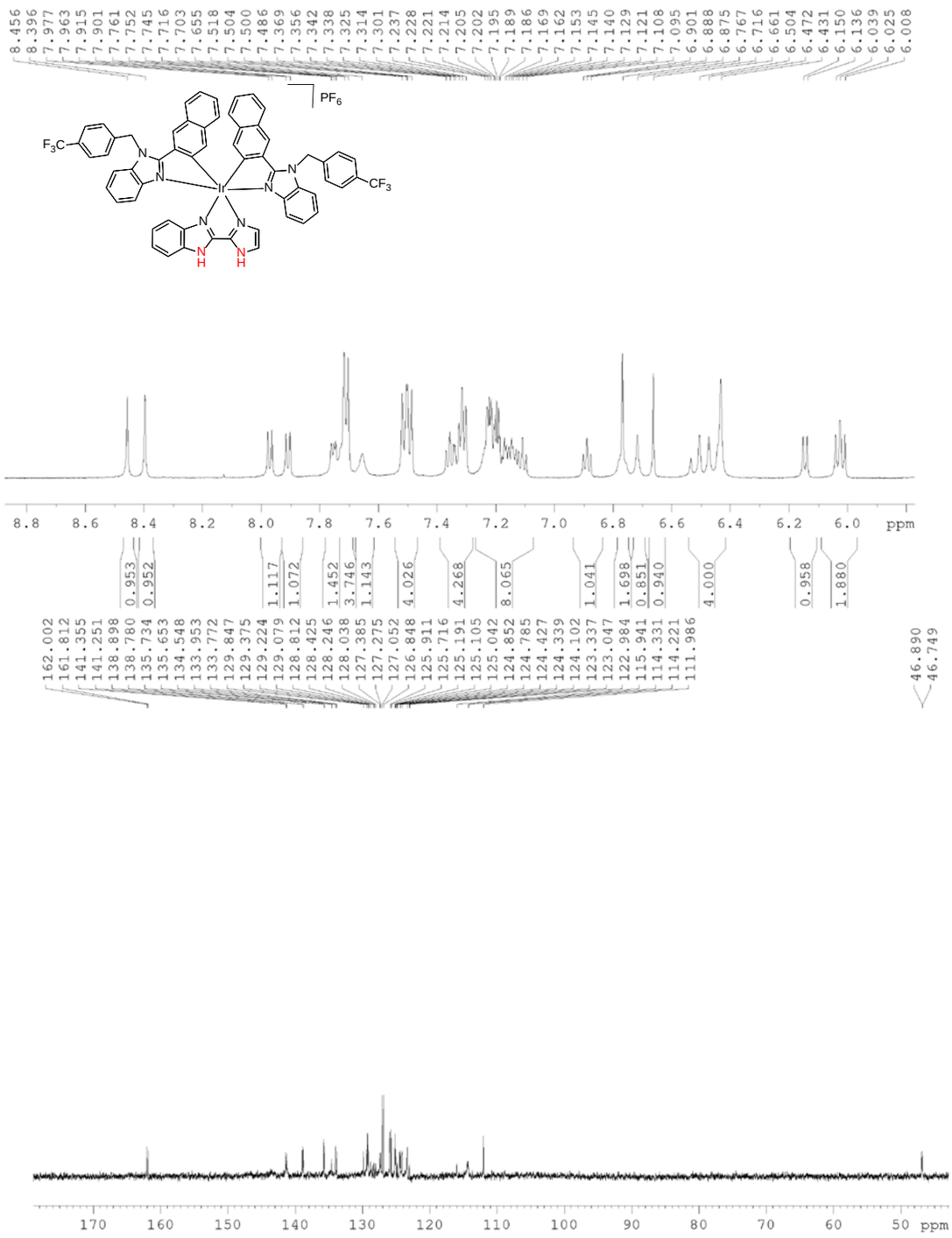


Figure S2. ^{1}H and ^{13}C NMR in [D_6]DMSO for **2**.

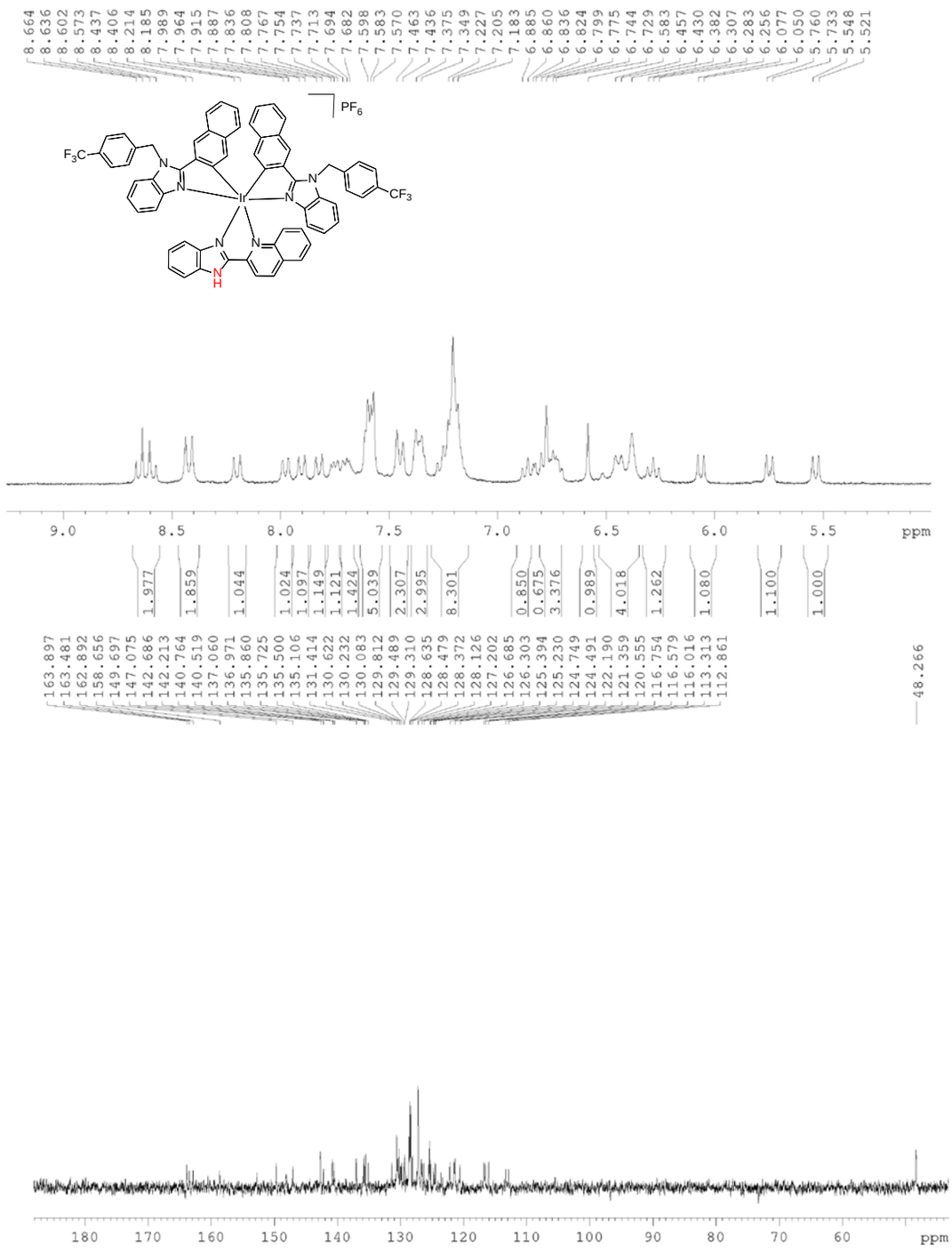


Figure S3. ^{1}H and ^{13}C NMR in [D_6]DMSO for **3**.

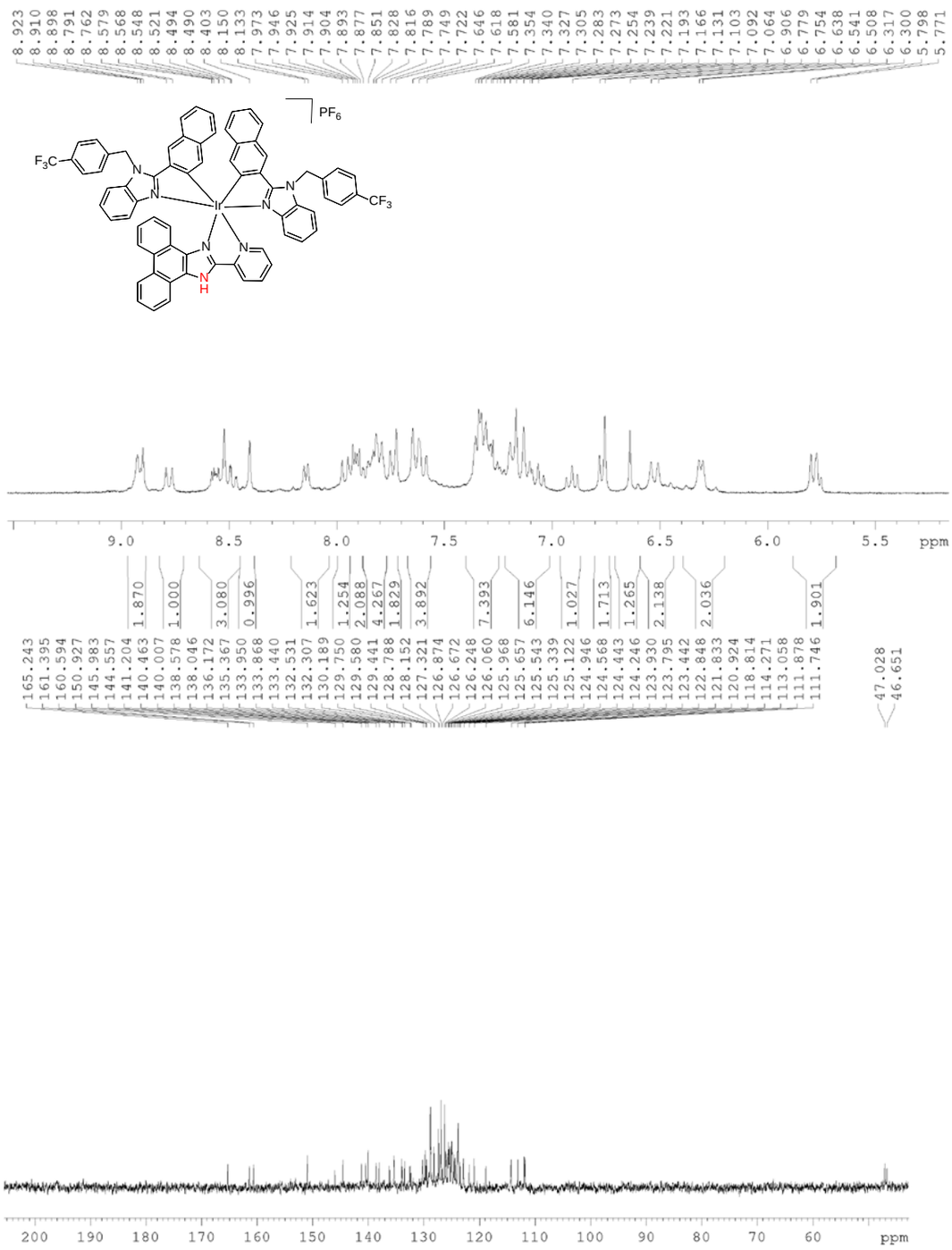


Figure S4. ^{1}H and ^{13}C NMR in [D_6]DMSO for **4** at 70ºC.

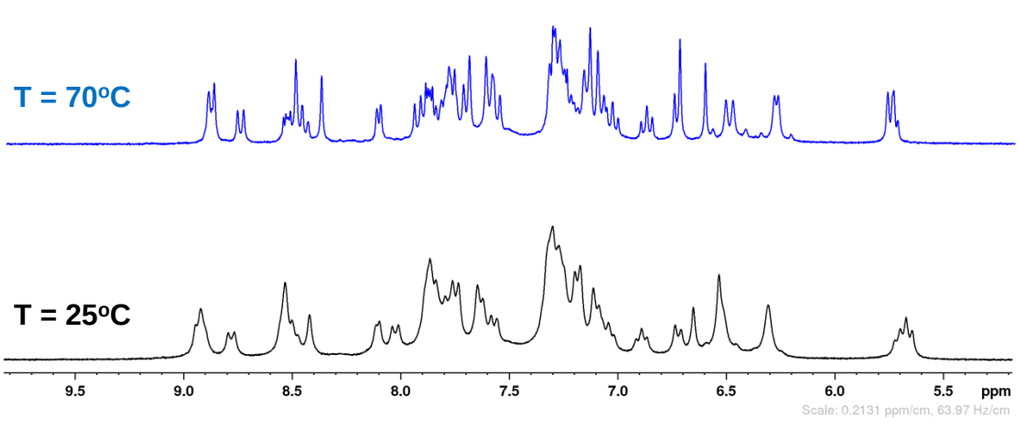


Figure S5. ^{1}H NMR in [D_6]DMSO for **4** at 70ºC (top) and 25ºC (bottom).

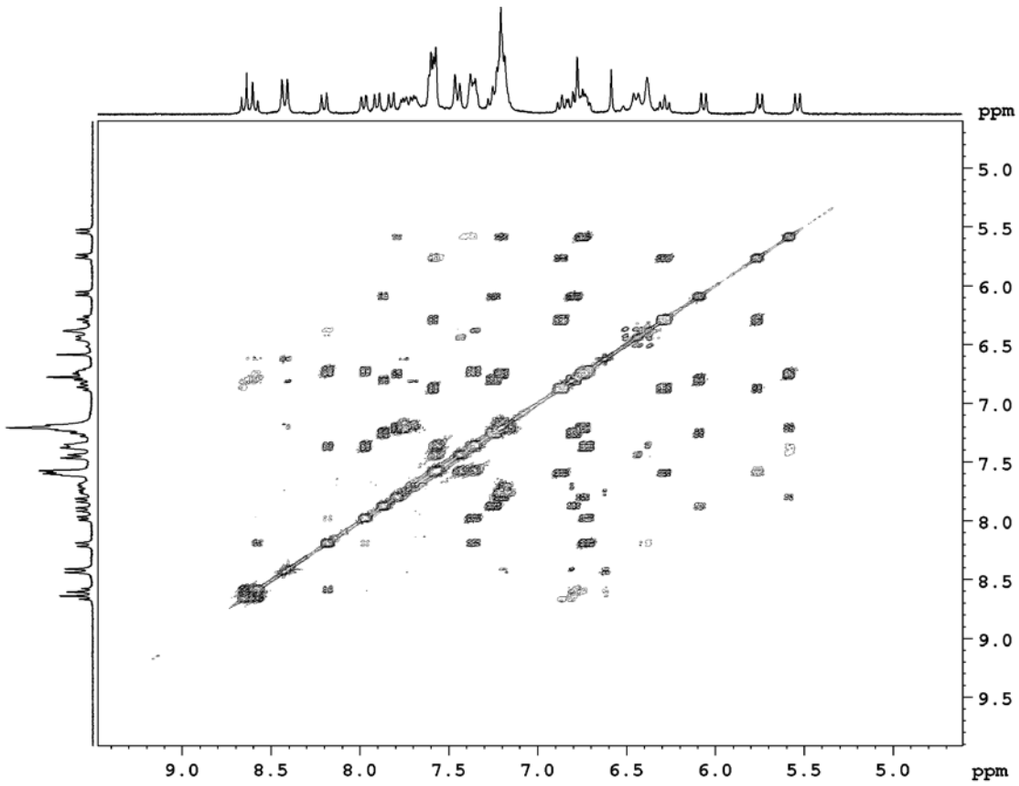


Figure S6. COSY 2D ^{1}H-^{1}H NMR of complex **3**.

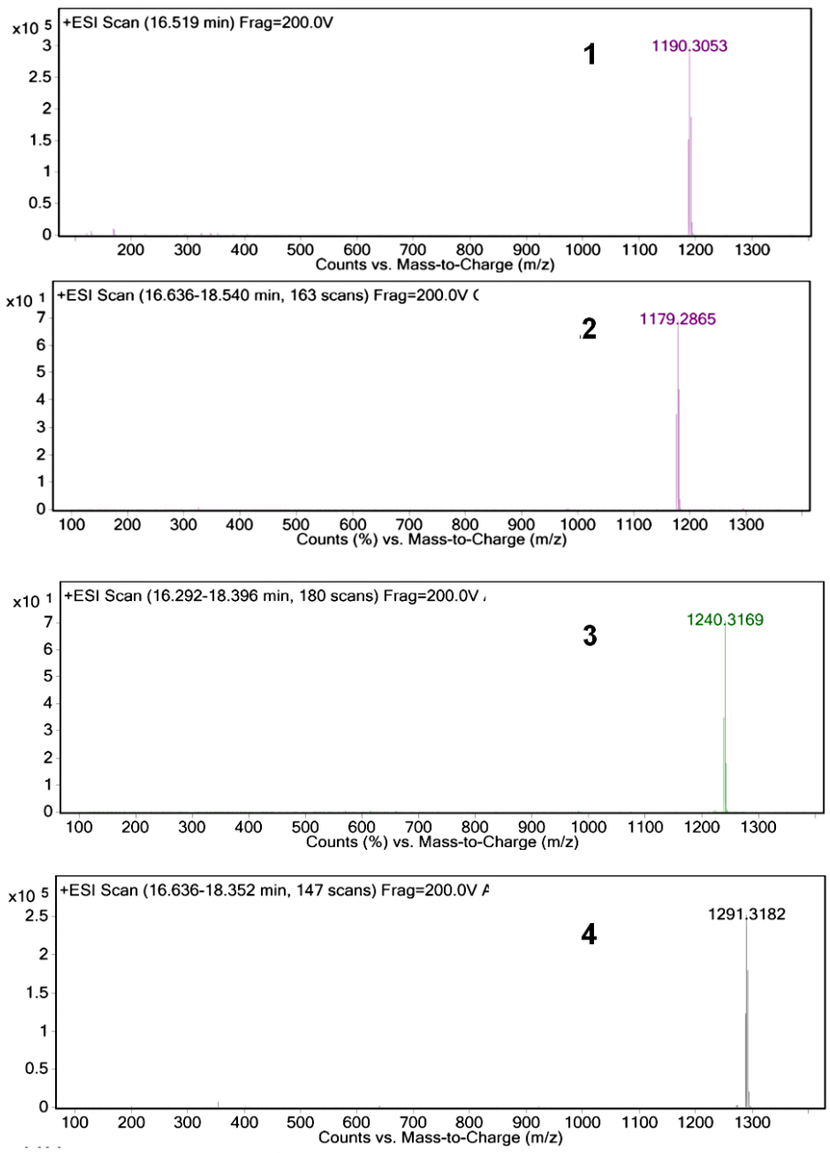


Figure S7. Positive-ion ESI-MS of complexes **1-4**.

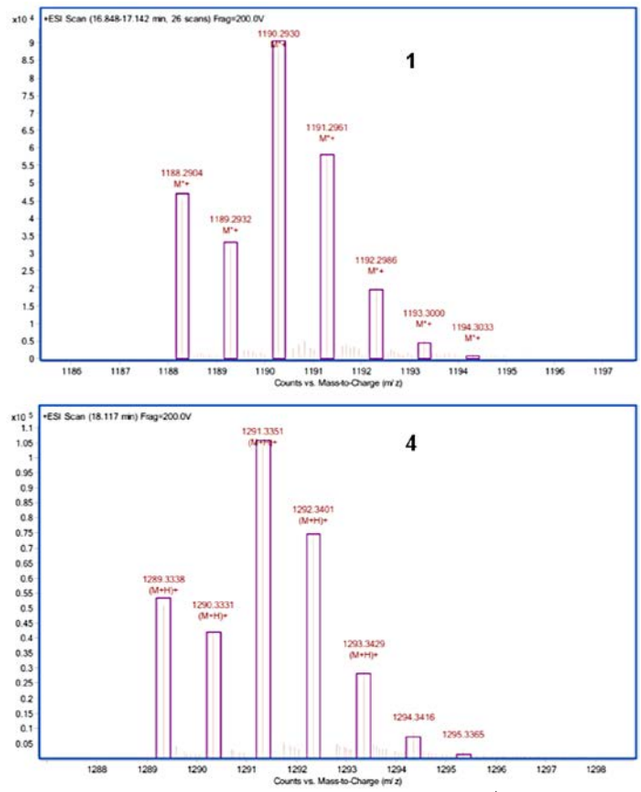


Figure S8. Theoretic vs experimental molecular ion $[M\text{-}PF_6]^+$ of **1** and **4**.

Table S1. HPLC method

Time (min)	0.1% formic acid in dH_2O	0.1% formic acid in CH_3CN
0-14	90	10
14-19.5	10	90
19.6-24	90	10

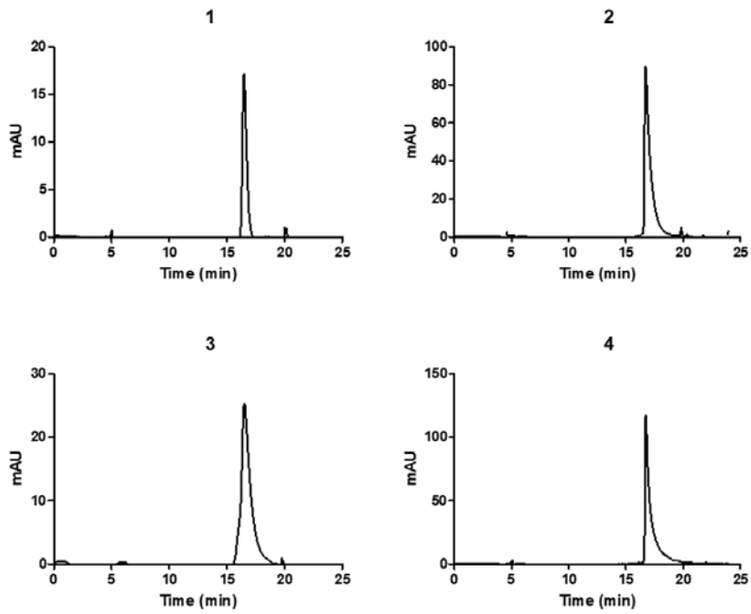


Figure S9. HPLC chromatograms of **1-4** in DMSO.

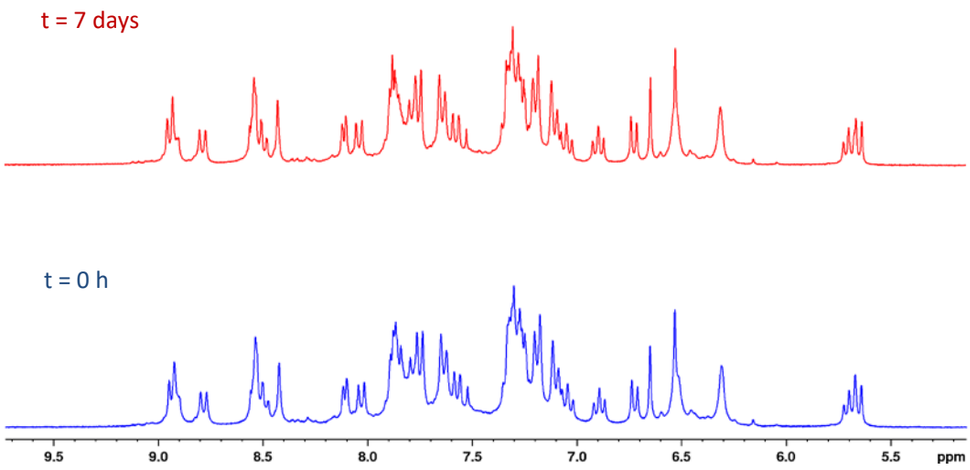


Figure S10. ^{1}H NMR spectra of **3** measured after dissolving immediately in DMSO[D_6] (bottom) and after 7 days (up) at RT.

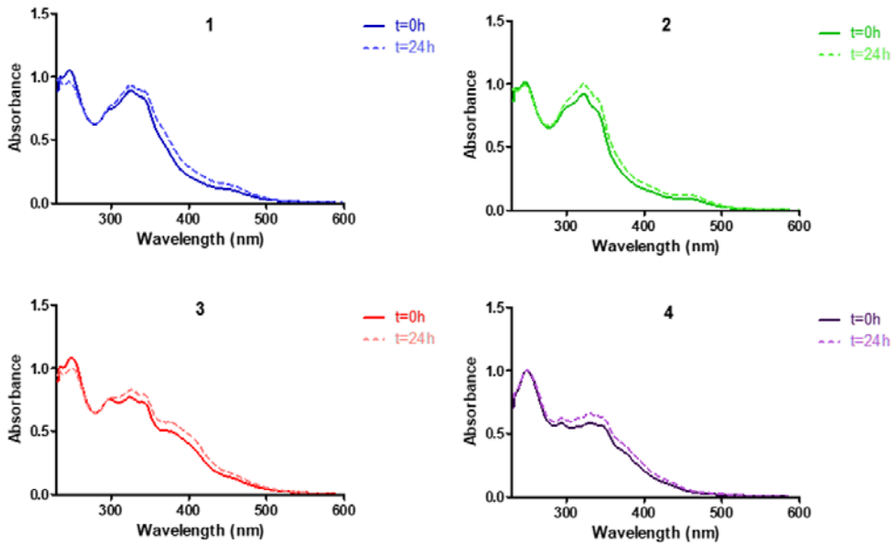


Figure S11. UV-Vis spectra of complexes **1-4** showing the stability in RPMI cell culture medium (1% DMSO).

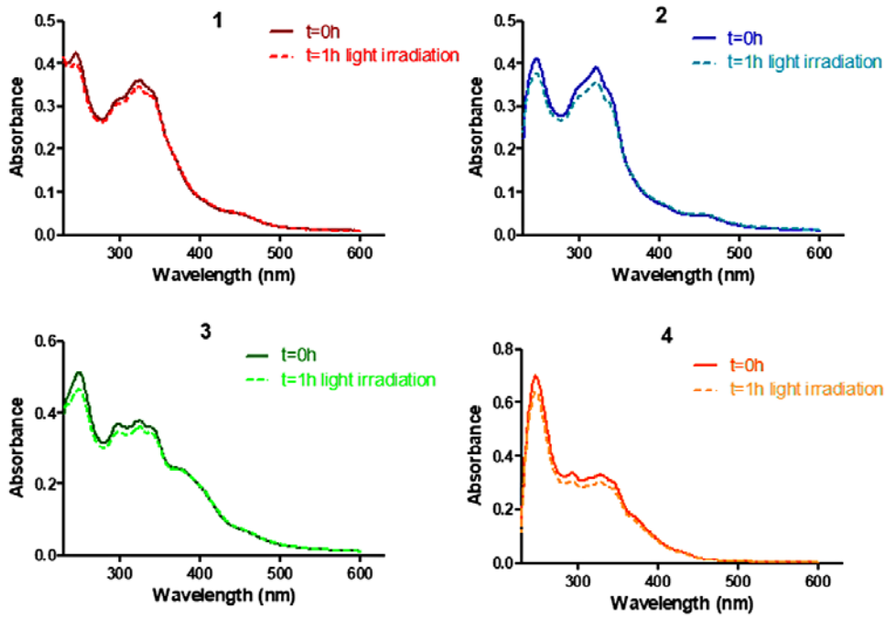


Figure S12. Photostability: UV-Vis spectra of complexes **1-4** before and after light exposure.

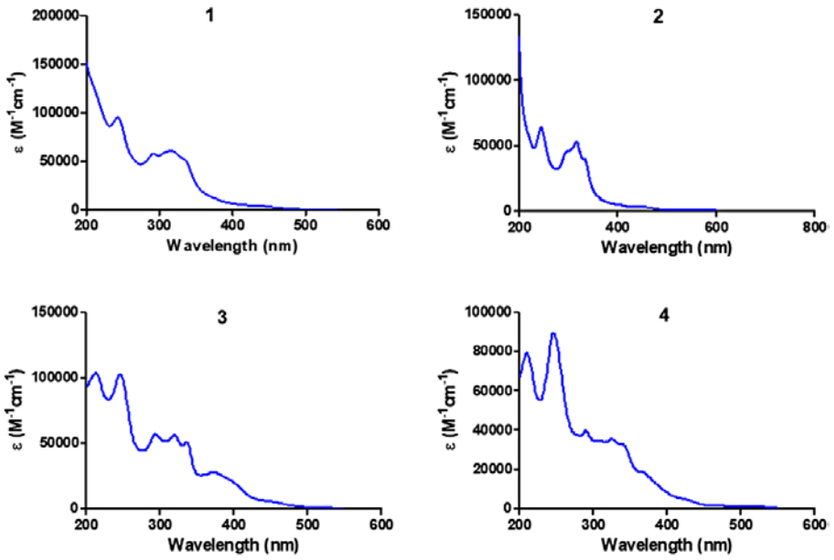


Figure S13. UV/Vis absorption spectra of **1-4** in acetonitrile.

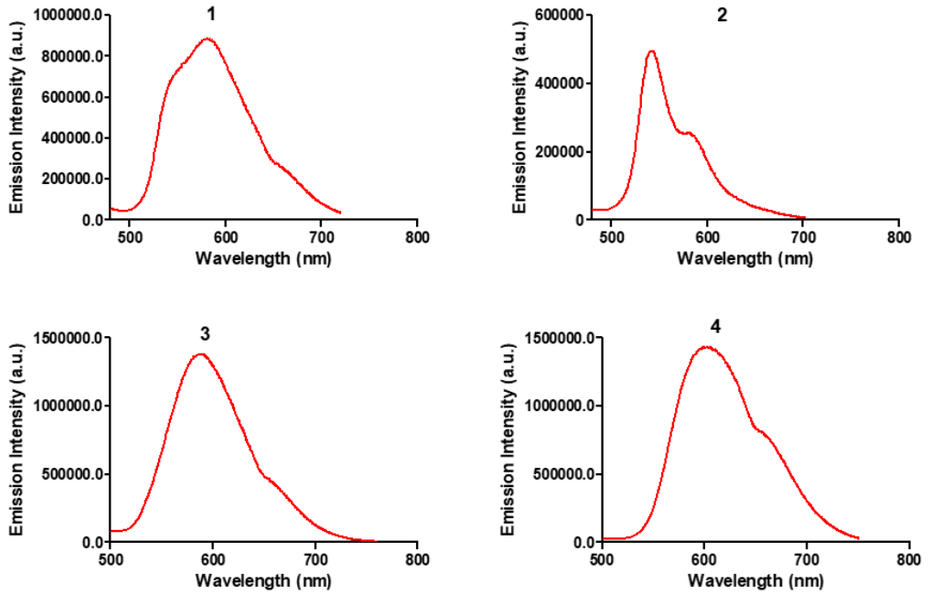


Figure S14. Emission spectra of complexes in aerated acetonitrile. λ_{exc}= 370, 360, 390 and 403 nm respectively.

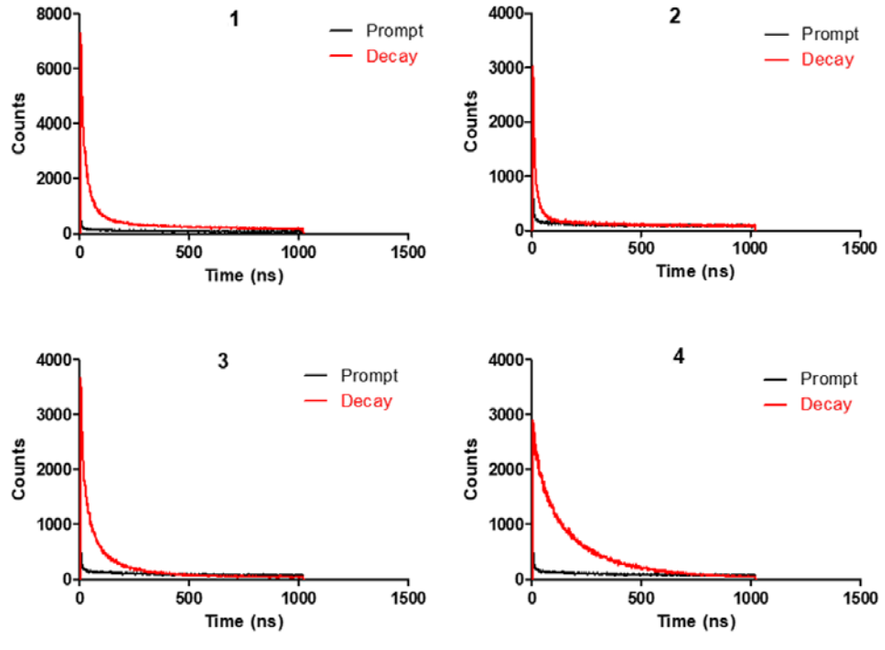


Figure S15. Emission decay kinetics of complexes in water (1% DMSO).

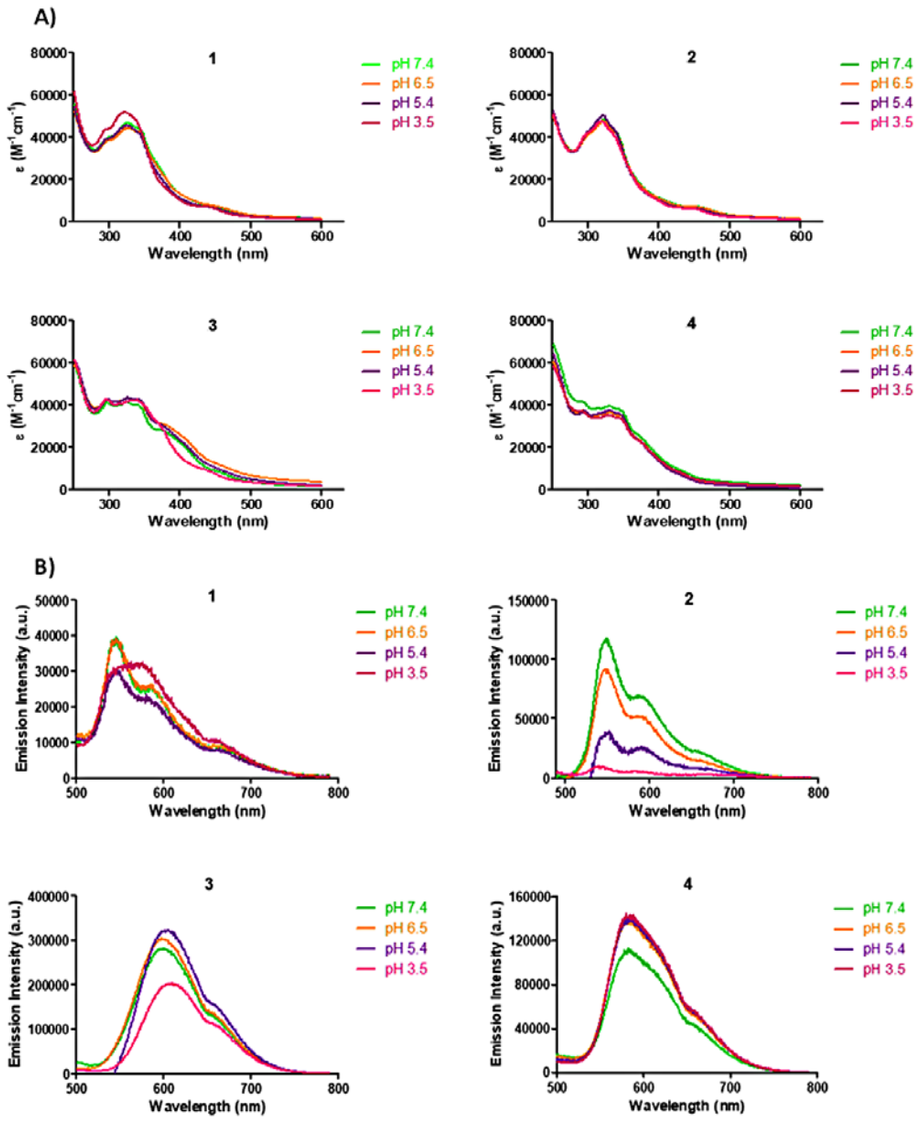


Figure S16. A) UV/Vis absorption spectra and B) emission spectra of **1-4** in buffer/DMSO (99:1) at different pH. The excitation wavelength was 405 nm.

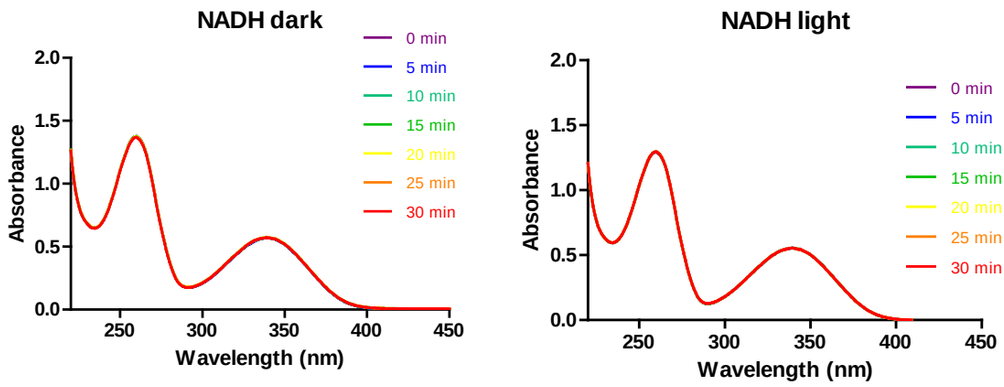


Figure S17. Stability of NADH in the dark and under light irradiation (465 nm, 8.4 mW/cm^2) after 30 min.

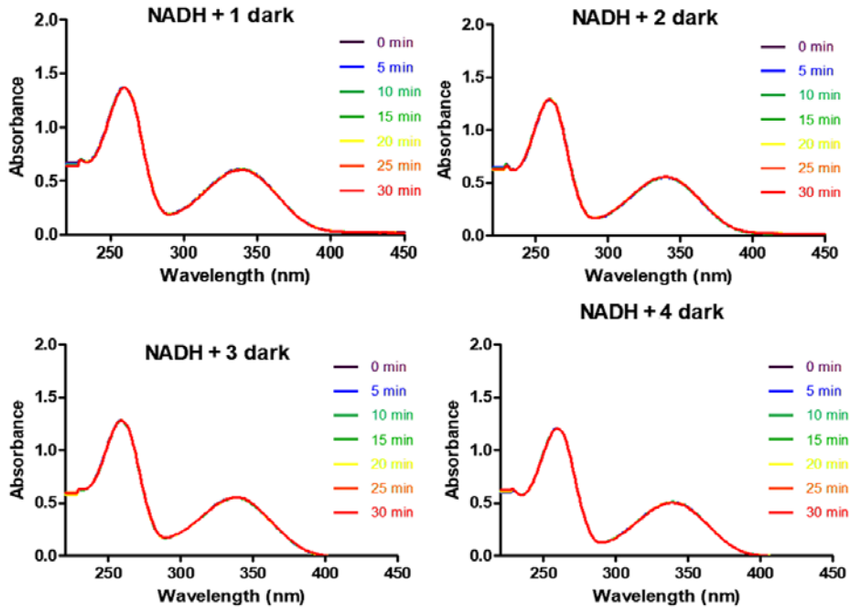


Figure S18. UV/Vis spectra of NADH (100 µM) in presence of complexes (5 µM) in the dark for 30 min.

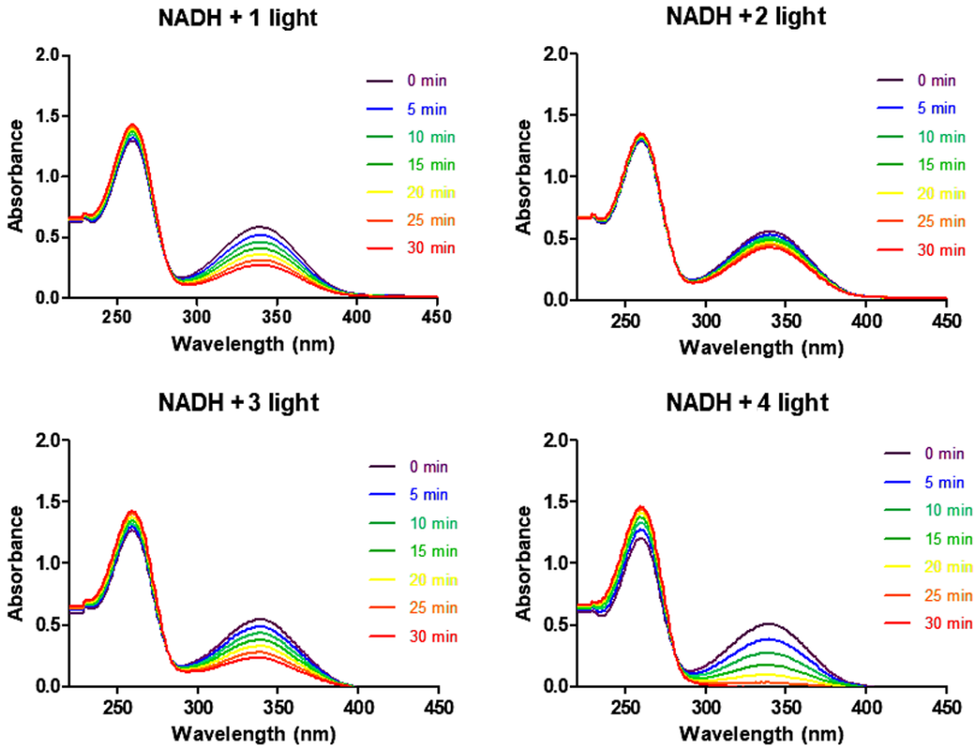


Figure S19. UV/Vis spectra of NADH (100 µM) in presence of complexes (5 µM) under 465 nm blue light irradiation for 30 min.

Table S2. TON and TOF of compounds **1-4**. TON was defined as the number of moles of NADH that Ir compound could convert in 30 min. TOF was calculated from the concentration of oxidized NADH (calculated by the difference of concentration of NADH) after 30 min divided by the concentration of complexes.

Compound	TON	TOF ($x10^4$) (s^{-1})	TOF (h^{-1})
1	10.1	55.9	20.1
2	4.2	23.2	8.3
3	9,9	55.5	20.0
4	16.4	91.3	32.9

Table S3. Log P values of **2a** measured using the ‘‘shake flask’’ method and the HPLC-method designed by Keppler *et al.*[7] at room temperature.

Method	φ_0	log *P*[a]
Shake flask (ICP-MS)	-	2.6 ± 0.1
HPLC-based method	76.7	2.3 ± 0.1

[a]Results are expressed as the mean ± SD from two independent experiments.

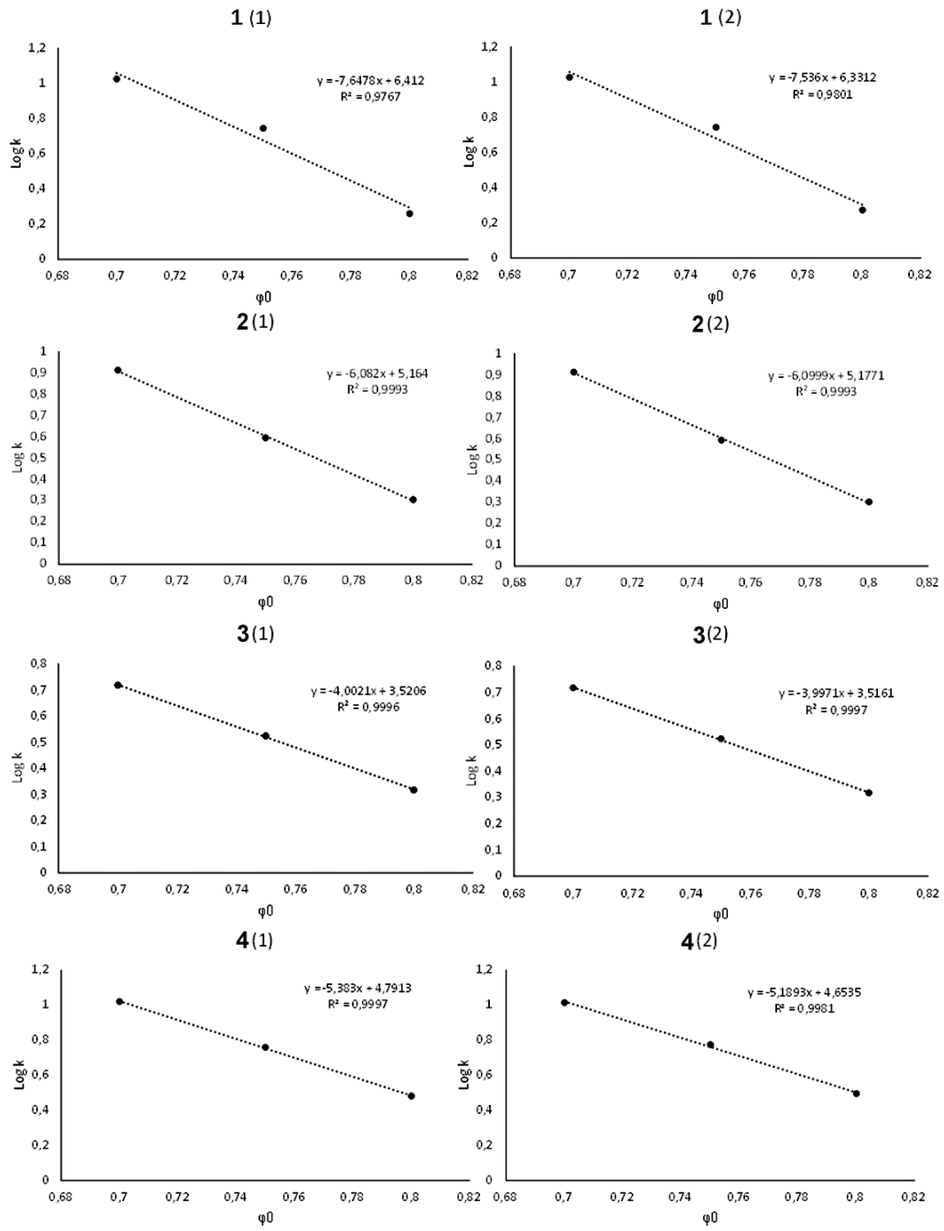


Figure S20. Soczewinski–Snyder relationship for **1-4** of two independent experiments.

Table S4. Mean chromatographic hydrophobicity parameter φ0 calculated using Soczewinski–Snyder relationship when Log k = 0.

Compound	φ_0
1	84
2	84
3	88
4	89

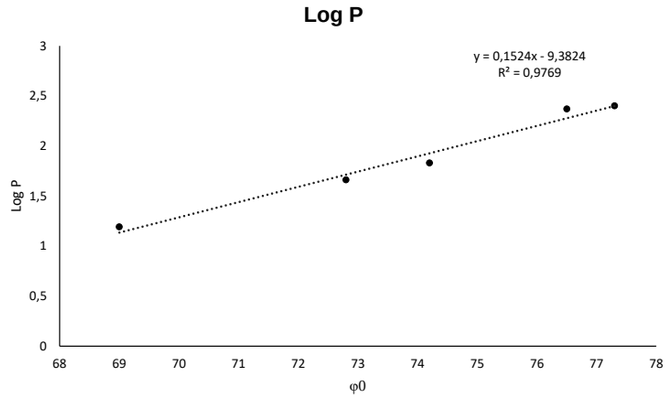


Figure S21. Graphic representation of the log *P* of the most lipophilic complexes found by Keppler *et al*. and their corresponding values of φ_0.

Table S5. IC_{50} values[a] (µM) obtained for HeLa cervical cancer cells treated with the investigated Ir complexes in the dark or after irradiation by visible light (1 h, λ_{max} = 420 nm, 77 ± 3 Wm^{-2}) determined by the MTT assay in normoxic or hypoxic conditions.

HeLa	**Normoxia**			**Hypoxia**		
	dark	420 nm	PI[b]	dark	420 nm	PI[b]
1	>300	13 ± 2	>22	>300	17±3	>17
2	>300	>50		>300	>50	
3	>300	9.7 ± 0.9	>31	>300	18±5	>17
4	>300	4.5 ± 0.6	>66	>300	6±2	>49

[a]Data represent the mean ± SD from three independent experiments, each made in triplicate. [b]PI (phototoxic index) = $[IC_{50}]_{dark}/[IC_{50}]_{420\ nm}$.

Table S6. IC_{50} values (µM) obtained for A375, 518.A2 melanoma and HeLa cervical cancer cells treated with the investigated Ir complexes in the dark or after irradiation by visible light (1 h, λ_{max} = 420 nm, 77 ± 3 Wm^{-2}) determined by Neutral Red assay in normoxic condition.

	A375		**518.A2**		**HeLa**	
	dark	**420 nm**	**dark**	**420 nm**	**dark**	**420 nm**
1	>50	16 ± 2	>50	11.4 ± 0.5	>50	20 ± 3
2	>50	>50	>50	48 ± 2	>50	>50
3	>50	13.8 ± 0.8	>50	8 ± 1	>50	17 ± 4
4	>50	5.9 ± 0.1	>50	3.3 ± 0.2	>50	6.5 ± 0.9

[a]Data represent the mean ± SD from two independent experiments, each made in triplicate.

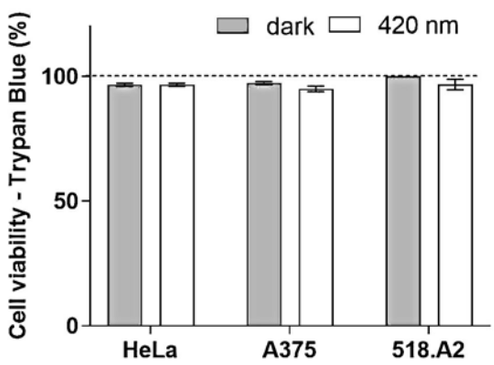


Figure S22. Viability of untreated cells non-irradiated (dark) or after irradiation by blue light for 1 h (420 nm) after 72 h recovery measured with the aid of trypan blue assay. Results are presented as mean ± SD from three independent experiments. Non-irradiated control cells were taken as 100%.

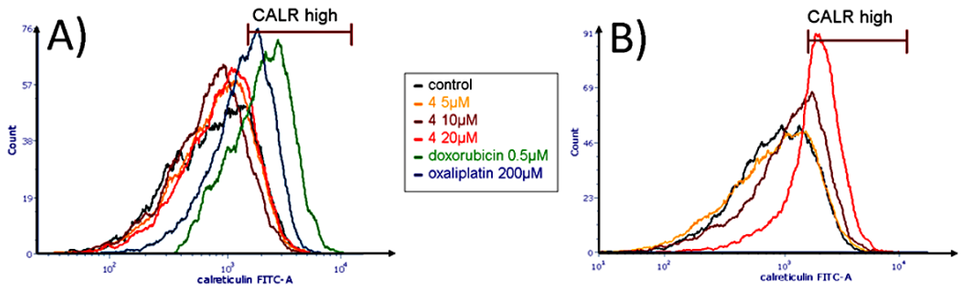


Figure S23. Representative flow cytometry histograms of ecto-Calreticulin exposure for A375 cells treated with the tested complexes (**4**, doxorubicin, oxaliplatin) in the dark (A) and after irradiation (B).

Table S7. Ecto-CALR exposing population (%) obtained from flow cytometry histograms.

	DARK	**IRRADIATED**
control	16.8	16.6
4 (5 µM)	14.7	17.2
4 (10 µM)	9.9	27.9
4 (20 µM)	16.6	60.1
doxorubicin (0.5 µM)	63.7	ND
oxaliplatin (200 µM)	41.2	ND

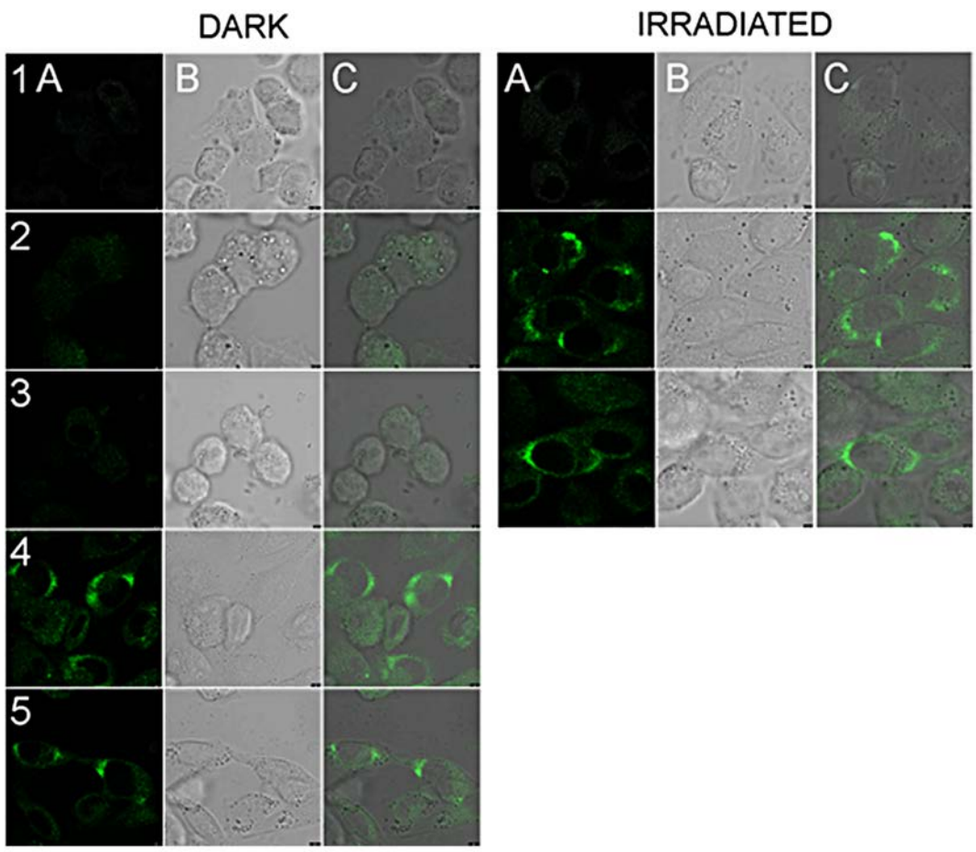


Figure S24. Immunofluorescence analysis of calreticulin exposure by confocal microscopy. A375 cells were untreated (panels 1) or treated with **4** (10 µM panels 2, 20 µM panels 3), doxorubicin (0.5 µM – panels 4) or oxaliplatin (200µM – panels 5) followed by irradiation with blue light (420nm, 1 h) or kept in the dark with subsequent incubation for 22 h in the drug-free medium in the dark. Channels: A. Fluorescence signal from CRT-Alexa fluor 488 conjugate. B. Bright field. C. Merge of the fluorescence and bright field channels.

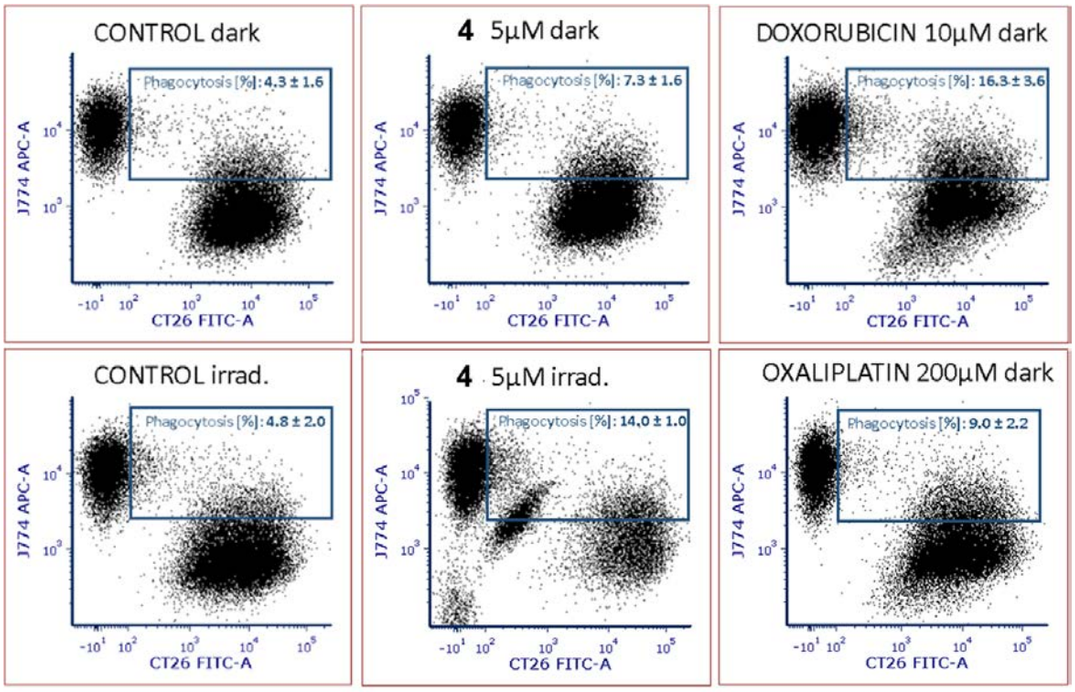


Figure S25. Phagocytosis assay CT26/J774.A1. Murine cancer cells CT26 (non-treated or pretreated with **4**, doxorubicin, or oxaliplatin) were stained with CellTracker™ green CMFDA and co-incubated with pre-stained (CellTracker™ red CMTPX) murine macrophages J774.A1. The cell mixture was analyzed by flow cytometry. Double-stained macrophages indicate phagocytosis.

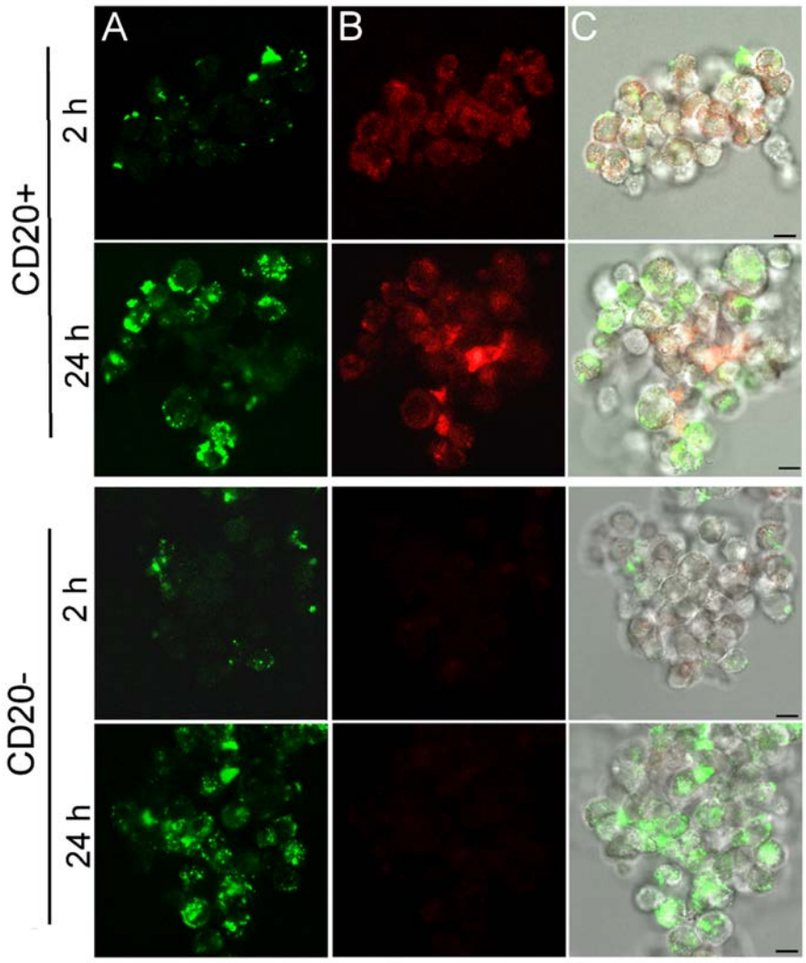


Figure S26. Penetration of complex **4** into 3D spheroids generated from A375CD20+ and A375CD20- cells. Cell spheroids (diameter: 40 – 60 µm) were treated with complex **4** (10 µM) and incubated for 2 h or 24 h in the dark. Spheroids were subsequently fixed and stained with APC anti-human CD20 antibody. Channels: Panels A, phosphorescence from complex **4**. Panels B, CD20 antibody. Panels C, overlay of the fluorescence channels and bright field. Scale bar represents 10 µm.

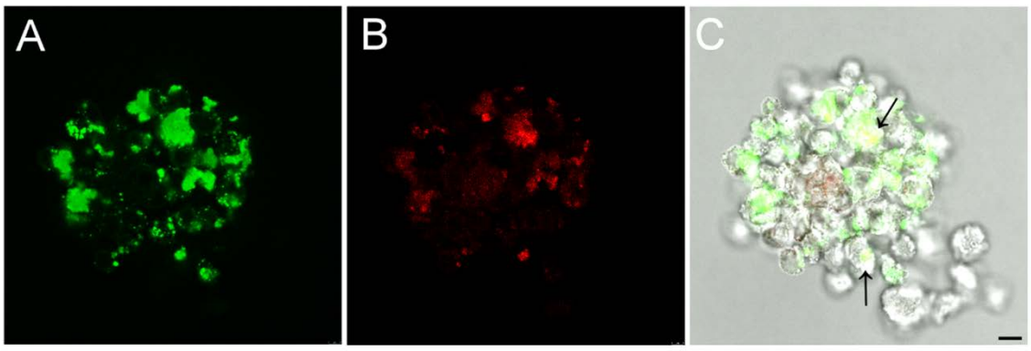


Figure S27. Penetration of complex **4** to 3D spheroid generated from unsorted A375 cells. Spheroids (diameter: 80 µm) were treated with complex **4** (10 µM) and incubated in the dark for 24 h. After washing, cells were co-stained with APC anti-human CD20 antibody (Miltenyi Biotec). Channels: Panel A, phosphorescence from complex **4.** Panel B, CD20 antibody. Panel C, overlay of the fluorescence channels and bright field. Arrows indicate the co-stained areas. Scale bar represents 10 µm.

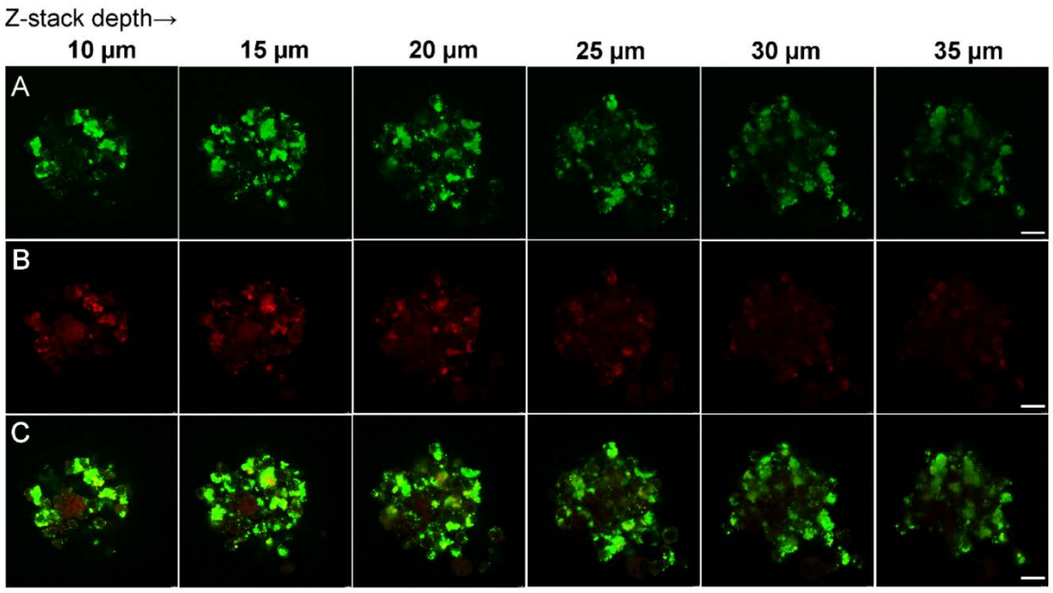


Figure S28. Penetration of complex **4** to 3D spheroid generated from unsorted A375 cells. Spheroids (diameter: 80 µm) were treated with complex **4** (10 µM) and incubated in the dark for 24 h. After washing, cells were co-stained with APC anti-human CD20 antibody (Miltenyi Biotec). Channels: Panels A, phosphorescence from complex **4.** Panels B, CD20 antibody. Panels C, overlay of the fluorescence channels. Confocal z-stacks were acquired in defined steps (10-35 µm). Scale bar represents 20 µm).

SMILES

1;FC(C(C=C1)=CC=C1CN2C3=C(C=CC=C3)[N]([Ir]456([N]7=C(C8=C6C=C(C=CC=C9)C9=C8)N(C%10=C7C=CC=C%10)CC(C=C%11)=CC=C%11C(F)(F)F)[N]%12=CC=CC=C%12C%13=[N]5C(C=CC=C%14)=C%14N%13)=C2C%15=C4C=C%16C(C=CC=C%16)=C%15)(F)F.FP(F)(F)(F)(F)F

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