Electronic Supplementary Material (ESI) for Inorganic Chemistry Frontiers.

Supporting information

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1. General Experimental procedures

1.1 Instrumentation

Mass spectra were recorded on a BRUKER ESQUIRE 3000 PLUS, with the electrospray (ESI) technique. ^{1}H and $^{13}C\{^{1}H\}$ NMR, including 2D experiments, were recorded at room temperature on a BRUKER AVANCE 400 spectrometer (^{1}H, 400 MHz, ^{13}C, 100.6 MHz) with chemical shifts (δ, ppm) reported relative to the solvent peaks of the deuterated solvent.[1] Steady-state photoluminescence spectra were recorded with a Jobin-Yvon-Horiba fluorolog FL-3-11 spectrometer using band pathways of 3 nm for both excitation and emission. UV/vis spectra were recorded with 1cm quartz cells on an Evolution 600 spectrophotometer. Quantum yields were measured using an absolute method provided by Hamamatsu Photonics Quantaurus-QY C11347-11.

1.2 Singlet oxygen production measurement

Singlet oxygen emission spectra were measured on a PicoQuant, FT300 fluorescence spectrometer equipped with a Hamamatsu H10330 A-45 thermoelectric cooled NIR-PMT unit with a spectral range of 950 nm to 1400 nm. Complexes **1** and **2** ($1.7\ 10^{-6}$M in CH_3CN) were excited with a 450 nm picosecond pulsed diode laser (P-C-450, PicoQuant) with 80 MHz repetition rate. Signals were digitised with a Time Harp 260 PCI card (PicoQuant). Spectra were recorded in the custom measurement mode of EasyTau software

1.3 Cell culture

A549 (human lung carcinoma) cell line was routinely cultured in high glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin. 4T1-luc2 cells were cultured in high glucose RPMI medium supplemented with 10% FBS without antibiotics. All cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2.

1.3 Antiproliferative activity assays

The MTT-reduction assay was used to analyse cell metabolic activity as an indicator for cell sensitivity to the complexes in A549 cell line. 10^5 cells/mL were seeded in complete

DMEM (0.5% FBS) medium in flat-bottom, 96-well plates (100 µl/well) and allowed to attach for 24 h prior to addition of compounds. Cells were incubated with complexes **1** and **2** for 24 or 48 h and then 10 µl of MTT (5 mg/mL in PBS) were added to each well and plates were incubated for 2 h at 37 °C. For PDT, the irradiation took place 5 or 24 hours after the addition of complexes, depending on the final incubation time, 24 or 48 hours, respectively. Finally, culture medium was removed and DMSO (100 µl/ well) was added to dissolve the formazan crystals. The optical density was measured at 550 nm using a 96-well multiscanner autoreader (ELISA) and IC_{50} was calculated using GraphPad Prims 5 or 9 software. Each compound was analyzed at least in three independent experiments.

1.4 Proliferation Assay in Lymphocytes

15 mL of Ficoll-Paque were placed in a 50 mL falcon, carefully 25 mL blood (from a blood bank not older than 8 hours) were added. Specifically, the Ficoll reagent is used for isolation of lymphocytes in high yield from peripheral blood using a simple and rapid centrifugation procedure. Therefore, the mixture was centrifuged at 1000 rpm for 30 minutes at 20ºC affording four layers, plasma on top, following peripheral blood mononuclear cells (PBMCs) and platelets, then next Ficoll Paque reagent (colorless) and red blood cells together with polymorphonuclear cell at the bottom. Thereafter, the upper layer was aspirated, without disturbing the interphase. The white central interface (PBMC) was carefully transferred to a new 50 mL conical tube, and 40 mL of PBS were added, mixed and centrifuged at 1000 rpm for additional 10 minutes. The supernatant was eliminated, and the resultant pellet was once again suspended in 40 mL of PBS and centrifuged for 10 minutes, twice. In this way, most of the platelets will remain in the supernatant upon centrifugation. So, the supernatant was removed, and the pellet was dissolved in DMEM. The extracted lymphocytes were counted and placed in both 96-well plates (12000 cells/well) for the antiproliferative assay (MTT assays following the same procedure as described in section 1.3) and in 6-well plates (150,000 lymphocytes/well) for the cytotoxicity assays.

The cytotoxicity assay was performed by flow cytometry. 150.000 lymphocytes were placed in 6-well plates and incubated with complexes 1 and 2 for 24 h. For PDT, the irradiation took place 5 h after the addition of complexes. After the incubation time, cells were washed with PBS (1 mL/well), and resuspended in 100 µL of a mixture of Annexin-binding

buffer (ABB 140 μM; NaCl 2.5 μM; CaCl2 10 μM; HEPES/NaOH pH 7.4), and FITC-conjugated Annexin V. They were incubated at room temperature in the dark for 15 minutes. Finally, cells were diluted to 500 μL with PBS and a total of 10,000 cells were acquired on a FACSCaliburTM flow cytometer.

1.5 Cell death mechanism assays

Apoptotic cell death pathway was determined by measuring phosphatidylserine exposure on cell surface in A549 cells. With this purpose, 10^5 cells/well were seeded in complete medium in flat-bottom, 6-well plates (2 mL/well) and left overnight to be attached to the bottom. Cells were treated for 48 h with synthetized complexes (**1** and **2**) in dark and under irradiation conditions (470 nm for 10 min) at concentrations of their IC_{50} and $2 \cdot IC_{50}$ values in duplicate. After the incubation time, cells were washed with PBS (1 mL/well), trypsinized (0.5 mL/well), and resuspended in 100 μL of a mixture of Annexin-binding buffer (ABB 140 μM; NaCl 2.5 μM; $CaCl_2$ 10 μM; HEPES/NaOH pH 7.4), and FITC-conjugated Annexin V and PI. They were incubated at room temperature in the dark for 15 minutes. Finally, cells were diluted to 500 μL with PBS and a total of 10,000 cells were acquired on a FACSCaliburTM flow cytometer. Cell death was analyzed using CellQuest Pro, FlowJo 7.6.1 and GraphPad Prism 9 software.

1.5 Fluorescence confocal microscopy

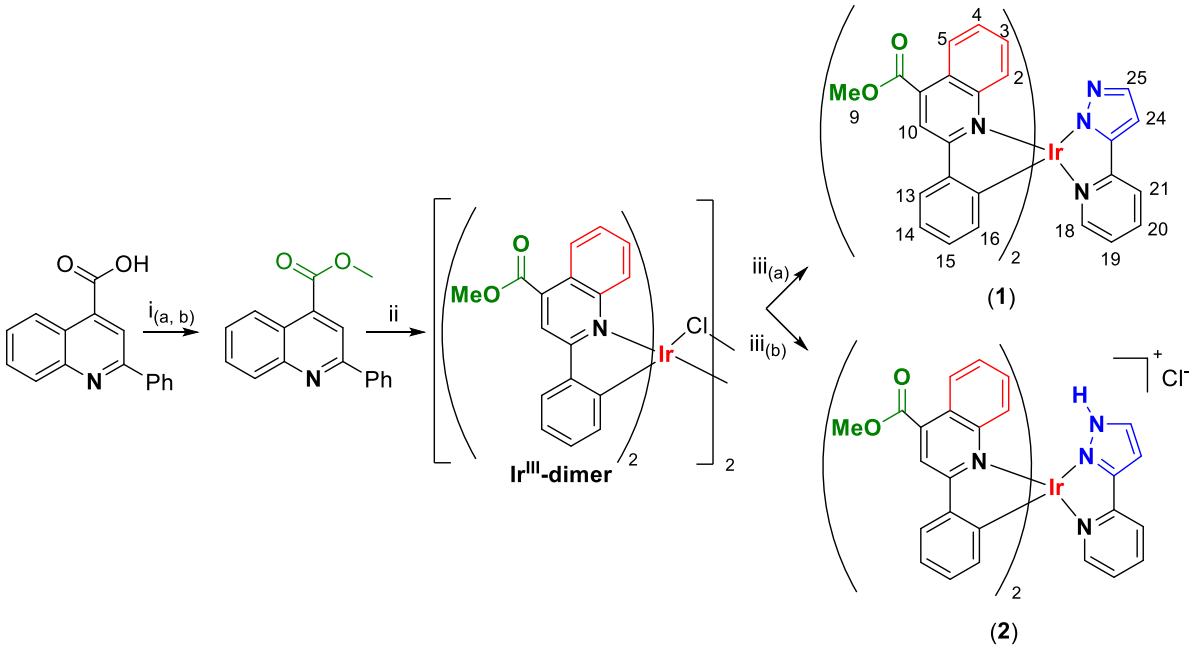
10^4 cells/well (A549, 4T1-luc2 cells) were seeded in complete medium in μ-slide 8 well (300 μl/well) and left 24 h to be attached to the bottom. Then, 200 μl of culture medium was removed and 100 μl of a solution of the corresponding complexes were added to a final concentration of 2 μM. The complexes were incubated with the cells for 2 hours. Thereafter, LysoTracker Green (LTG) at 100 nM, CellBlue Tracker at 500 nM were added. They were incubated with the cells for 45 LTG and 30 min CBT, at room temperature. One that the quenching effect was demonstrated, LTG was added just before starting the fluorescence microscopy visualization. Eventually the medium was replaced with fresh medium. Images were collected in a sequential mode in a ZEISS LSM 880 confocal microscope with a 60x oil immersion lens, a line average of 4, and a format of 1024x1024 pixels using excitation wavelength of either 405 (CBT) → 407-480nm , 488 nm (LTG)→ 500-560nm, and

561 nm (complexes) → 575-715nm. The confocal pinhole was 1 Airy unit. Images were analyzed with Zen Blue Little software.

1.6 **Cellular ROS assay**

Reactive oxygen species (ROS) levels in A549 cells were determinated using the DCFDA/H2DCFDA - Cellular ROS Assay Kit (ab113851, Abcam), following the manufacturer's instructions. Briefly, $5 \cdot 10^4$ cells/well were seeded in flat-bottom, 24-well plates (500 µl/well) in complete DMEM medium and 24 hours later the compounds were added at the indicated concentrations. These were incubated for 24 hours, and some preparations were irradiated at 470 nm for 10 min after 5 hours from the addition of the complexes. Finally, the cells were incubated with 100 µl of ROS working solution (20 µM) at 37 ºC for 30 min and analysed by flow cytometry. A total of 10,000 cells were acquired on a FACSCaliburTM flow cytometer (BD Biosciences) and ROS levels were analysed with CellQuest Pro (BD Biosciences) and FlowJo 10.8.1 (Becton Dickinson [BD]) softwares.

2. Synthetic procedures and in vitro assays



Scheme S1: Synthetic pathway for the synthesis of Iridium (III) dimmer precursor and complexes **1** and **2**. i_a) $SOCl_2$, MeOH, (0 °C); i_b) reflux, 2 h; ii) $IrCl_3 \cdot nH_2O$, etoxyethanol, reflux, 24 h; $iii_{(a)}$ 3-(2-pyridyl)pyrazole, Cs_2CO_3, DCM, 318 K, 20 h; $iii_{(b)}$ DCM:MeOH (1:1), 298 K, 20 h.

2.1a. Synthesis of methyl 2-phenylquinoline-4-carboxylate.[2]

2-Phenylquinoline-4-carboxylic acid (5.4 mmol) was solved in methanol (10 mL) and cooled to 0–5 °C. Thionyl chloride (8.2 mmol) was added drop-wise under stirring. Once the addition was over, the reaction mixture was refluxed for 2 h, affording a yellow solution. The methanol and the excess of thionyl chloride were removed. The residue was neutralized using a solution of sodium bicarbonate (50 mL). The aqueous layer was extracted with chloroform (3 × 20 mL); the combined organic layer was dried over anhydrous sodium sulfate and dried to get the desired compound as pale yellow solid (1.12 g, 85%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.72 (ddd, J = 8.6, 1.5, 0.6 Hz, 1H), 8.35 (s, 1H), 8.22 – 8.14 (m, 3H), 7.71 (ddd, J = 8.4, 6.9, 1.5 Hz, 1H), 7.60 – 7.40 (m, 4H), 4.00 (s, 3H) ppm.

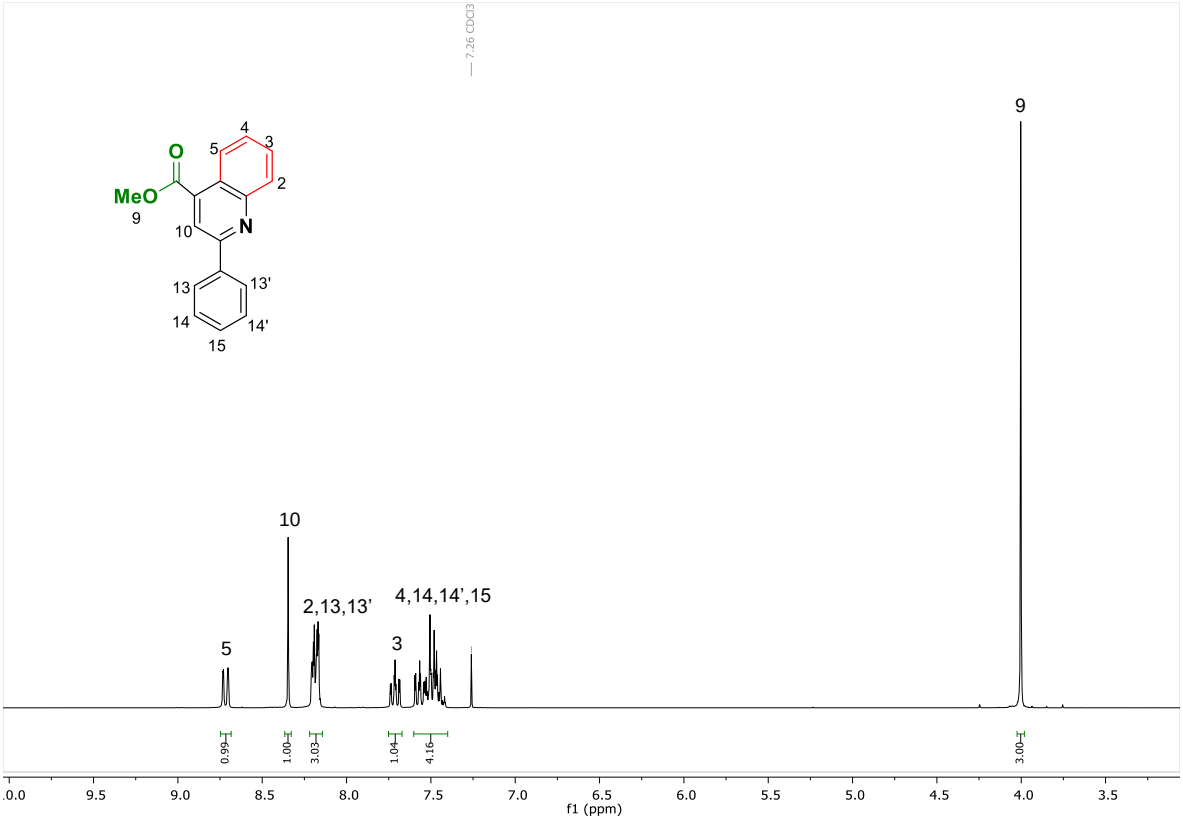


Figure S1. 1H NMR (400 MHz, $CDCl_3$, 25 °C) spectrum of ligand.

2.1b. Synthesis of the Ir^{III} dimmer, $[Ir(mpc)_2(\mu\text{-}Cl)]_2$.

200 mg (0.67 mmol) of methyl 2-phenylquinoline-4-carboxylate (mpc) were disolved in the minimum volume of 2-etoxiethanol (5ml) and $IrCl_3 \cdot nH_2O$ (0.34 mmol) was added. The reaction mixture was refluxed at 383 K for 24 h. The resultant suspension was cooled and

filtered affording the desired complex as a dark red solid, which was dried under vacuum. (164 mg; 0.11 mmol; 64 %).

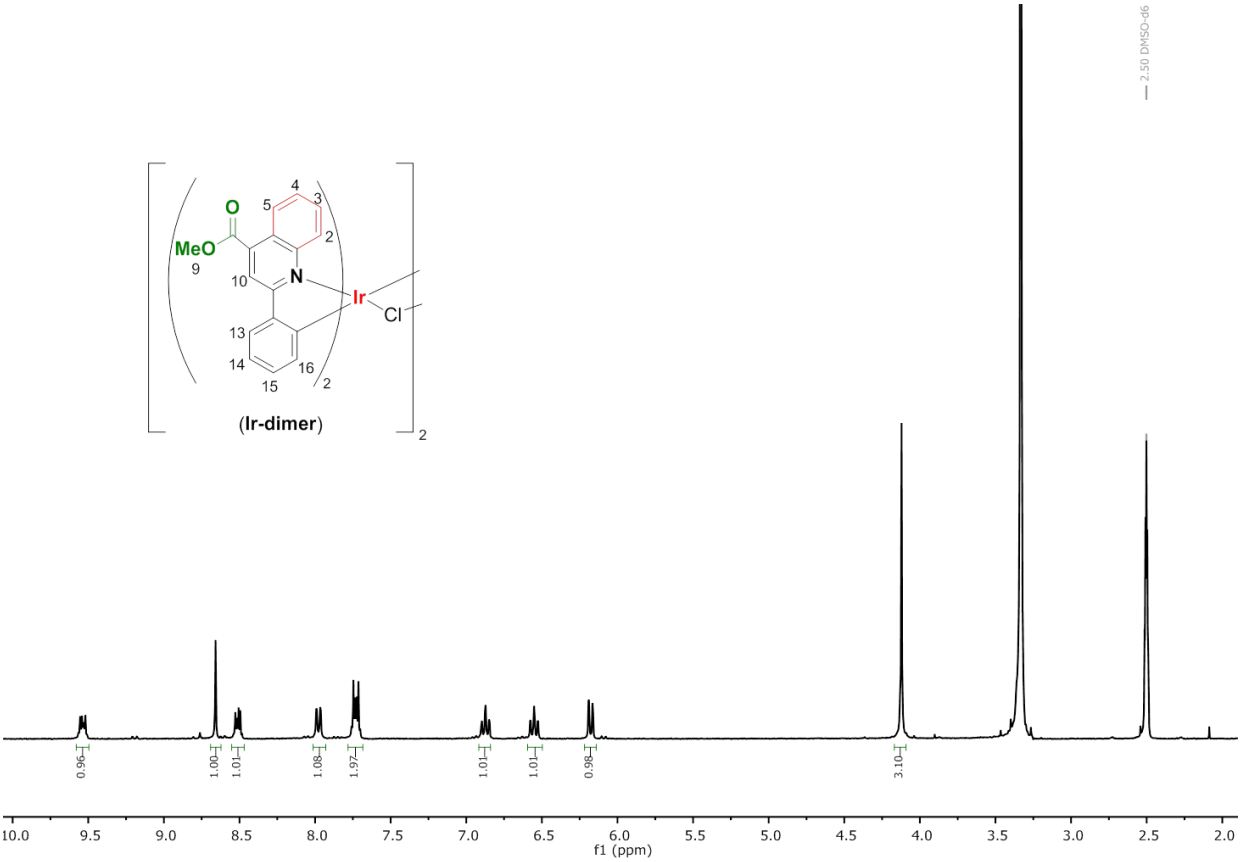


Figure S2. 1H NMR (400 MHz, DMSO-d^6, 25 °C) spectrum of Ir-dimmer.

2.2. Synthesis of complex 1 and characterisation

50 mg of iridium dimmer precursor (0.033 mmol) were solved in 5 ml of dry dichloromethane. Once all the dimmer has been solubilized, 9.85 mg (0.066 mmol) of 2-(1H-pyrazol-3-yl)pyridine and 15 mg of K_2CO_3 were added. The resultant dark red suspension was stirred and refluxed at 318 K for 20 h. The bright red residue was filtered through zelite, and the filtered was collected and dried. The red solid obtained was purified by column chromatography using as eluents a mixture of DCM/MeOH (9/1). (25.8 mg; 0.03 mmol; 91 %)

^{1}H NMR (400 MHz, DMSO-d6) δ 8.73 (s, 1H, H10), 8.66 (s, 1H, H10'), 8.31 – 8.23 (m, 3H, H5,5',13), 8.12 – 8.09 (m, 1H, H13'), 7.96 (d, J = 8.9 Hz, 1H, H18), 7.70 – 7.58 (m, 3H, H2,2',19), 7.51 – 7.41 (m, 3H, H4,4',21), 7.21 (d, J = 1.9 Hz, 1H, H25), 7.11 (dddd, J = 8.6, 7.2, 6.4, 1.3 Hz, 2H, H3',14), 7.07 – 6.98 (m, 3H, H3,14',20), 6.81 (td, J = 7.4, 1.2 Hz, 1H, H15), 6.67 – 6.59 (m, 2H, H15',16), 6.37 (d, J = 1.9 Hz, 1H, H24), 6.31 (dd, J = 7.8, 1.2 Hz, 1H,H16'), 4.05 (d, J = 4.6 Hz, 6H, H9,9') ppm. ^{13}C NMR (101 MHz, DMSO-d6) δ 195.0, 185.5, 170.5, 170.0, 165.7, 165.7, 158.9, 155.0, 152.6, 148.3, 148.2, 147.7, 146.5, 146.3, 145.7, 140.0, 138.5, 138.2, 137.5, 135.2, 133.4, 130.5, 130.2, 130.0, 129.4, 127.5, 127.2, 126.7, 125.8, 125.7, 125.1, 123.2, 123.0, 121.9, 121.1, 119.1, 118.0, 117.8, 103.5, 54.9, 53.81 ppm. Found: C, 54.2; H, 3.7, N, 7.8%. Calc. for $C_{42}H_{30}IrN_5O_4 \cdot 3H_2O$: C, 55.1; H, 3.8; N, 7.65% HRMS (ESI): m/z (calcd.) =861.1927, m/z (found, 1+H) = 862.1988.

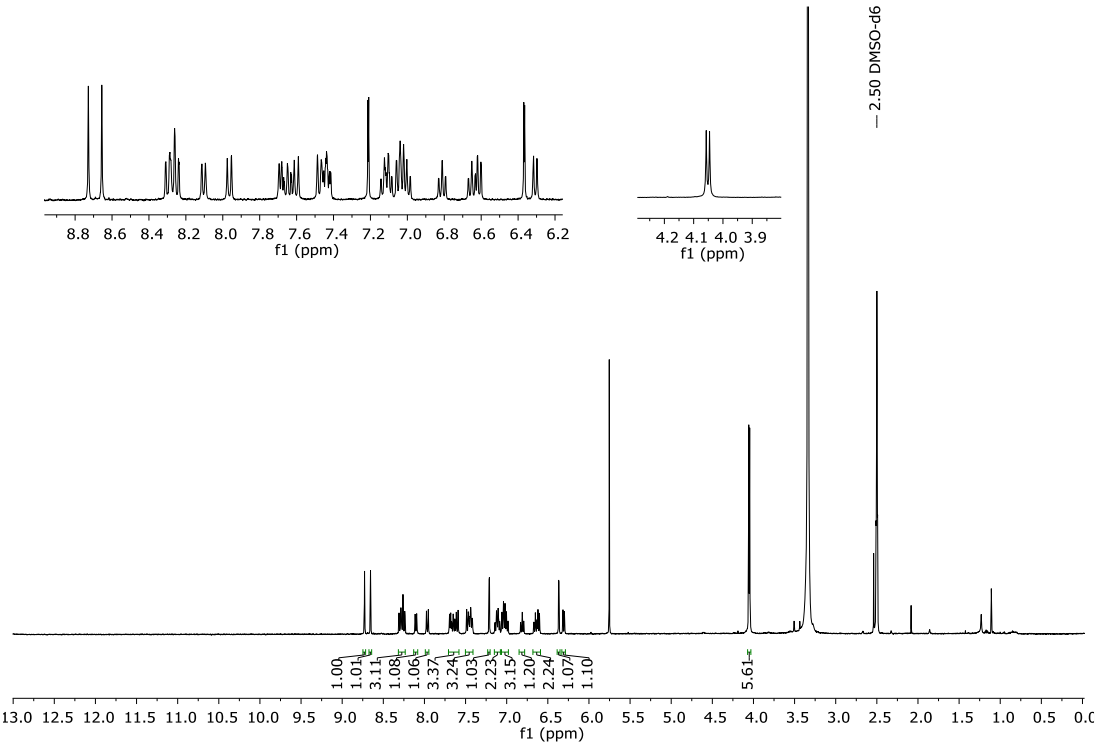


Figure S3. ^{1}H NMR (400 MHz, DMSO-d_6, 25 °C) spectrum of complex **1**.

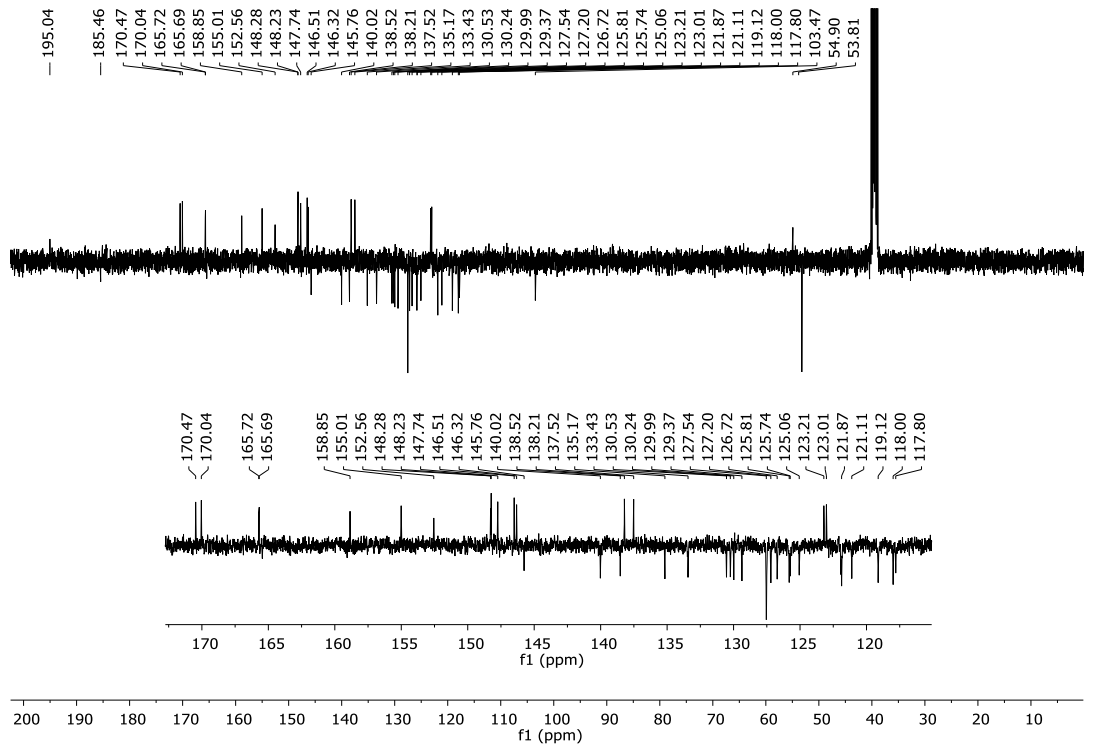


Figure S4. APT NMR (101 MHz, DMSO-d_6, 25 °C) spectrum of complex **1**.

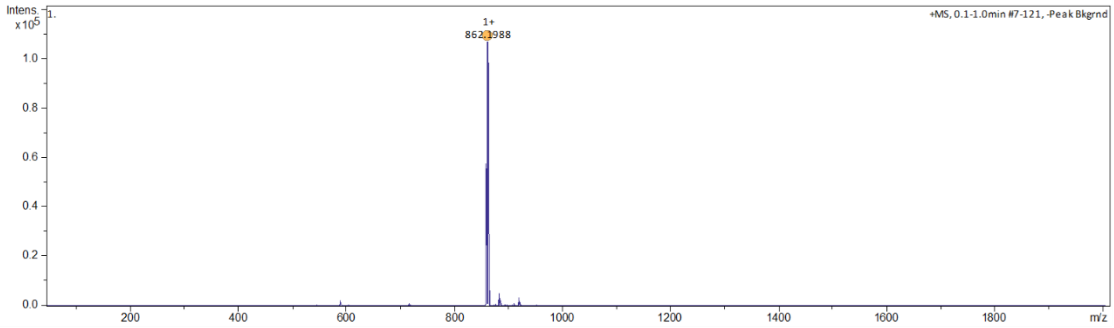


Figure S5. HRMS (ESI +) of complex **1**.

2.3. Synthesis of complex 2 and characterisation

50 mg of iridium dimmer precursor (0.033 mmol), and 9.85 mg (0.066 mmol) of 2-(1H-pyrazol-3-yl)pyridine were solved in 5 ml of a mixture of dry dichloromethane / methanol (1/1). The mixture was stirred at r.t. for 2 h. The resultant bright red solution was refluxed at 325 K for 20 h. The solvents were removed under vacuum until minimum volume, and the product was precipitated with cool pentane, affording a red solid which was filtered and dried. Column chromatography using as eluents a mixture of DCM/MeOH (9/1) was necessary to purified the desired complex. (21.7 mg; 0.25 mmol; 76 %)

^{1}H NMR (400 MHz, DMSO-d6) δ 13.96 (s, 1H, H26), 8.81 (s, 1H, H10), 8.74 (s, 1H, H10'), 8.38 – 8.30 (m, 3H, H5,5',13), 8.23 (d, J = 8.0 Hz, 1H, H13'), 8.01 – 7.85 (m, 4H, H2,3,18,25), 7.65 – 7.45 (m, 4H, H2',4,4',19), 7.31 (dd, J = 21.1, 8.5 Hz, 1H, H21), 7.23 (t, J = 7.0 Hz, 1H, H3')7.19 (t, J = 7.6 Hz, 1H, H14), 7.13 (t, J = 7.5 Hz, 1H, H14'), 7.07 (t, J = 7.9 Hz, 1H, H20), 6.99 (s (sb), 1H, H24), 6.86 (t, J = 7.5 Hz, 1H, H15), 6.81 (t, J = 7.6 Hz, 1H, H15'), 6.54 (d, J = 7.9 Hz, 1H, H16), 6.38 (d, J = 7.7 Hz, 1H, H16'), 4.07 (d, J = 5.9 Hz, 6H, H9,9') ppm. ^{13}C NMR (101 MHz, DMSO-d6) δ 186.5, 170.3, 169.6, 165.5, 165.4, 151.2, 147.3, 147.2, 146.8, 145.9, 145.8, 145.6, 139.9, 139.0, 138.7, 134.3, 133.9, 131.1, 131.0, 130.7, 130.6, 128.0, 127.8, 126.3, 126.1, 124.9, 123.4, 123.2, 122.9, 122.6, 118.3, 118.0, 111.9, 105.2 53.4, 53.4 ppm. HRMS (ESI): m/z (calcd.) =862.2011, m/z (found) = 862.1979. Found: C, 53.4; H, 3.8, N, 7.7%. Calc. for $C_{42}H_{31}IrClN_5O_4 \cdot 2H_2O$: C, 54.0; H, 3.8; N, 7.5%

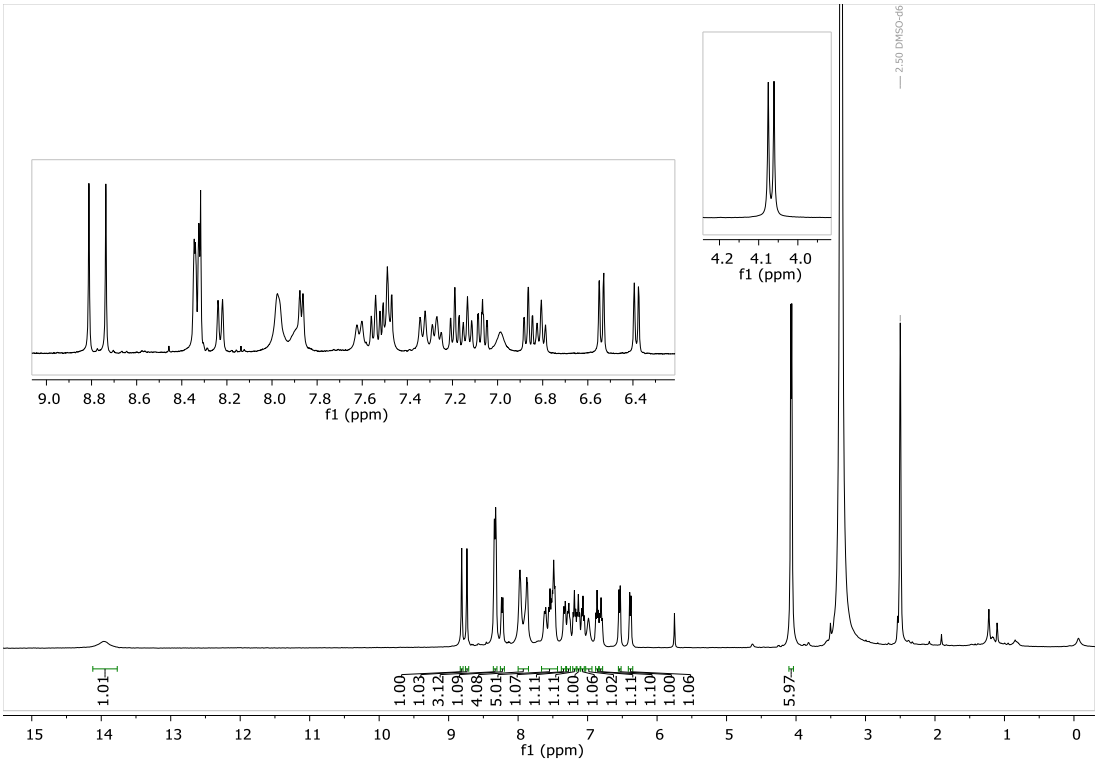


Figure S6. ^{1}H NMR (400 MHz, DMSO-d$_6$, 25 °C) spectrum of complex **2**.

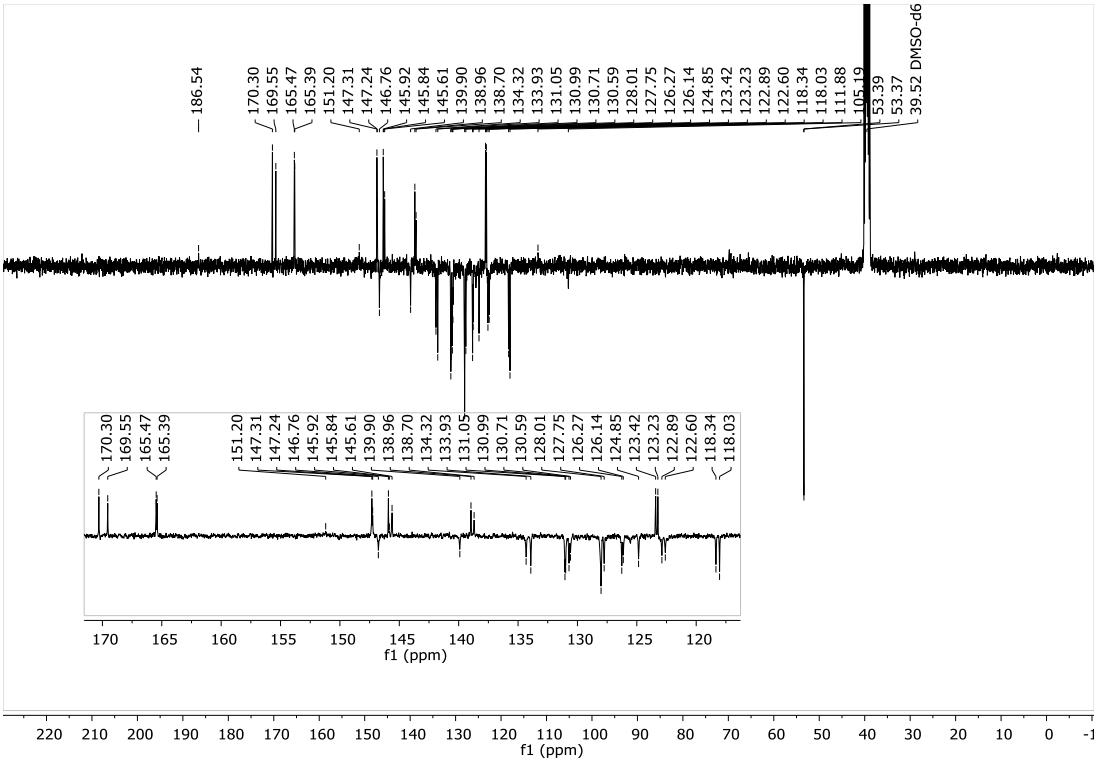


Figure S7. APT NMR (101 MHz, DMSO-d$_6$, 25 °C) spectrum of complex **2**.

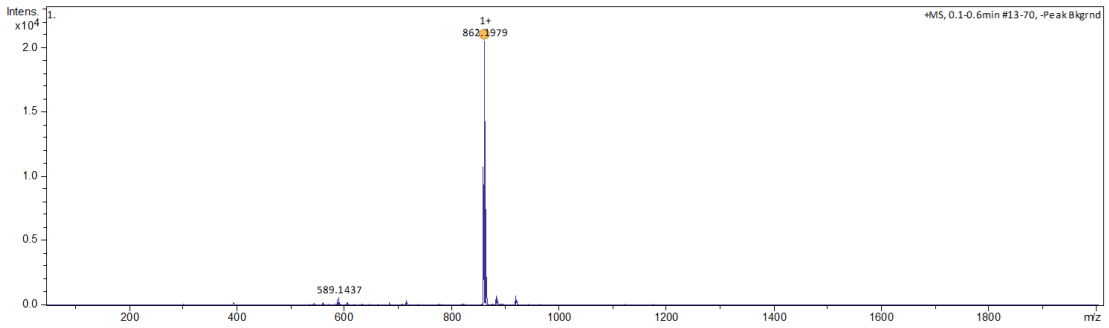


Figure S8. HRMS (ESI +) of complex **2**.

2.4. Optical Properties Analysis

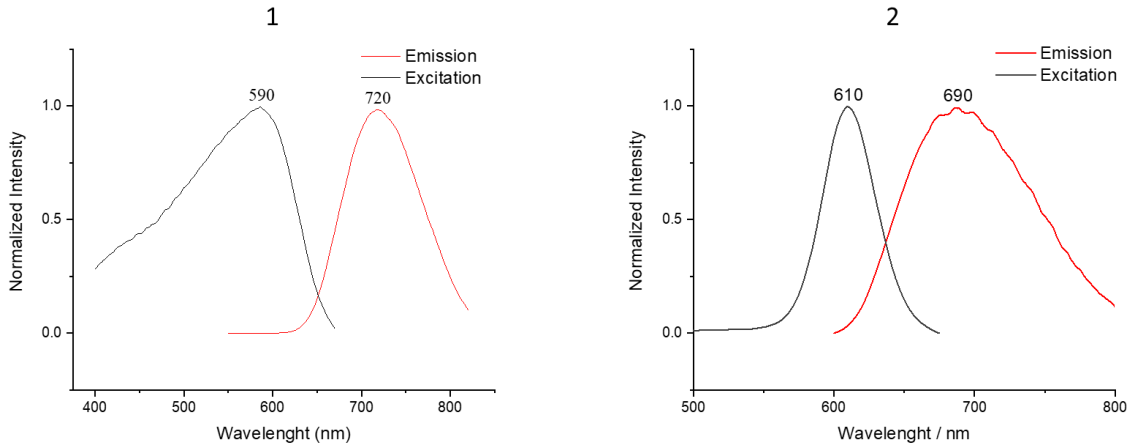


Figure S9. Emission and excitation spectra of complexes **1** and **2** (10^{-3}M), respectively, in DMSO solution at 298K .

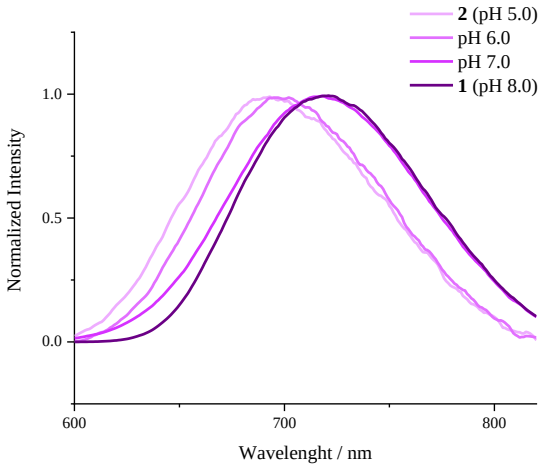


Figure S10. Change on emission maximum of complex **1** at different pH, until total conversion to complex **2**. Measurement conditions: to a solution 10^{-5} M of **1** in DMSO, HCl (10^{-5} M) was added drop-by-drop. After each addition, a pH-meter was used for adjusting the pH.

2.5. Cytotoxicity and photocytotoxicity assays

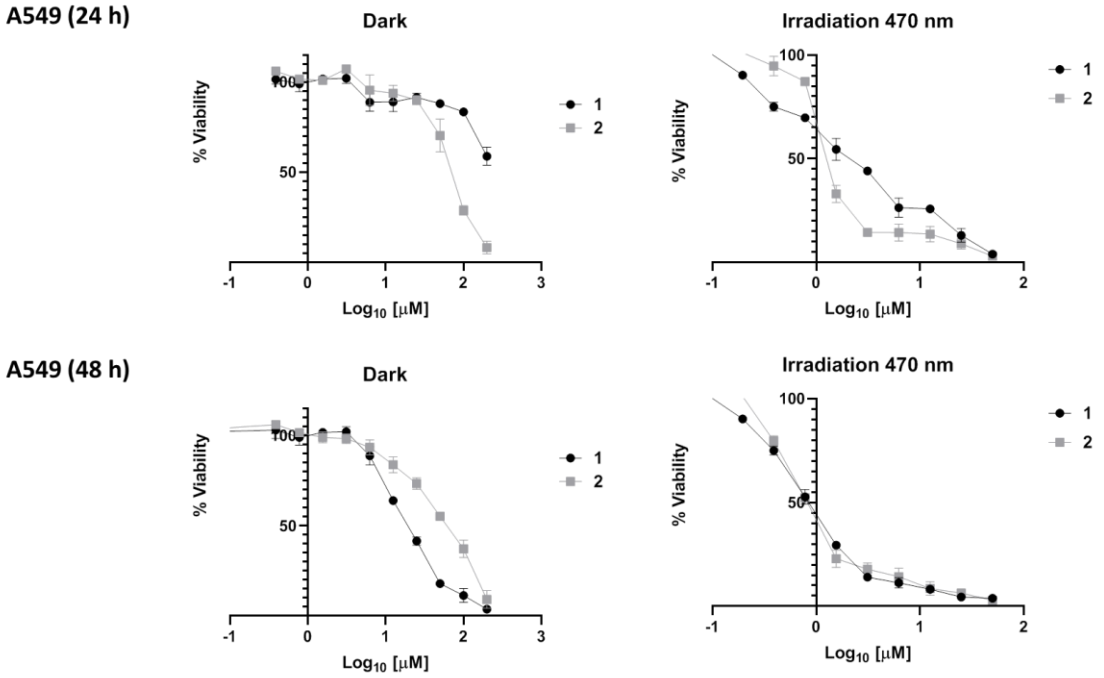


Figure S11. Plot of dose-response for complexes **1** and **2** in A549 at different conditions.

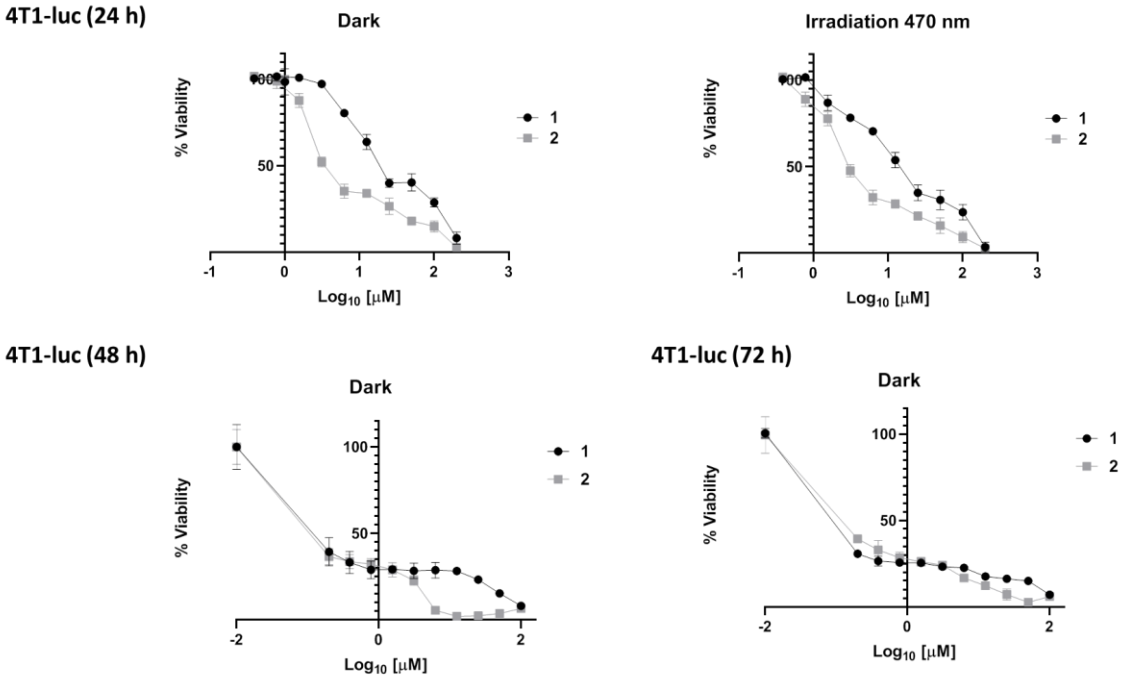


Figure S12. Plot of dose-response for complexes **1** and **2** in 4T1-luc at different conditions.

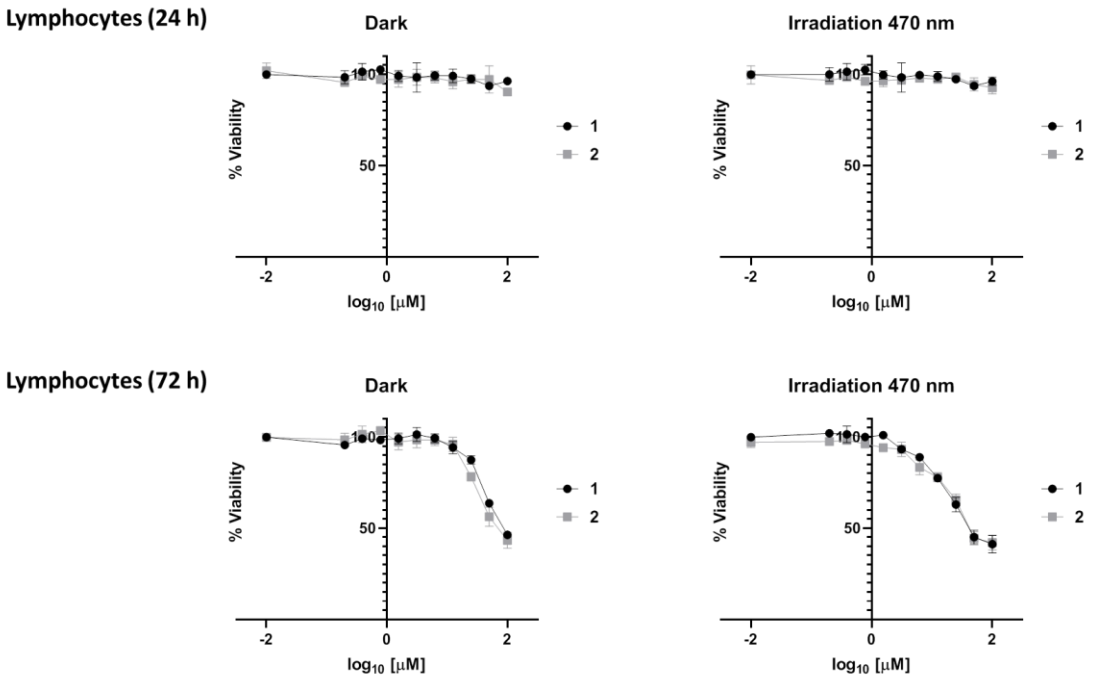


Figure S13. Plot of dose-response for complexes **1** and **2** in lymphocytes at different conditions.

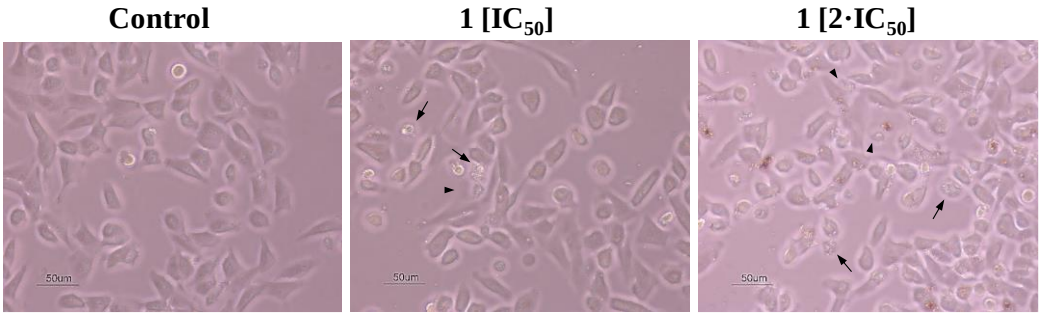


Figure S14. Cell microscopy images of **1** incubated with A549 for 24 h (irradiation at 470nm, 10 min). Black and blue arrows point to examples of apoptotic and necrotic cells respectively. Small triangles show cells containing cytoplasmatic vacuoles.

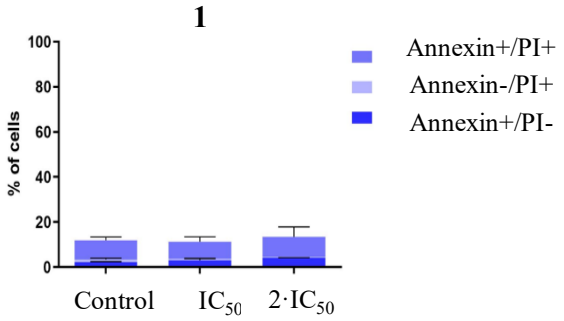


Figure S15. Flow cytometry graph of complex **1** incubated with A549 cells for 24 h and irradiation at 470 nm during 10 min using Annexin V-FTIC and PI as markers.

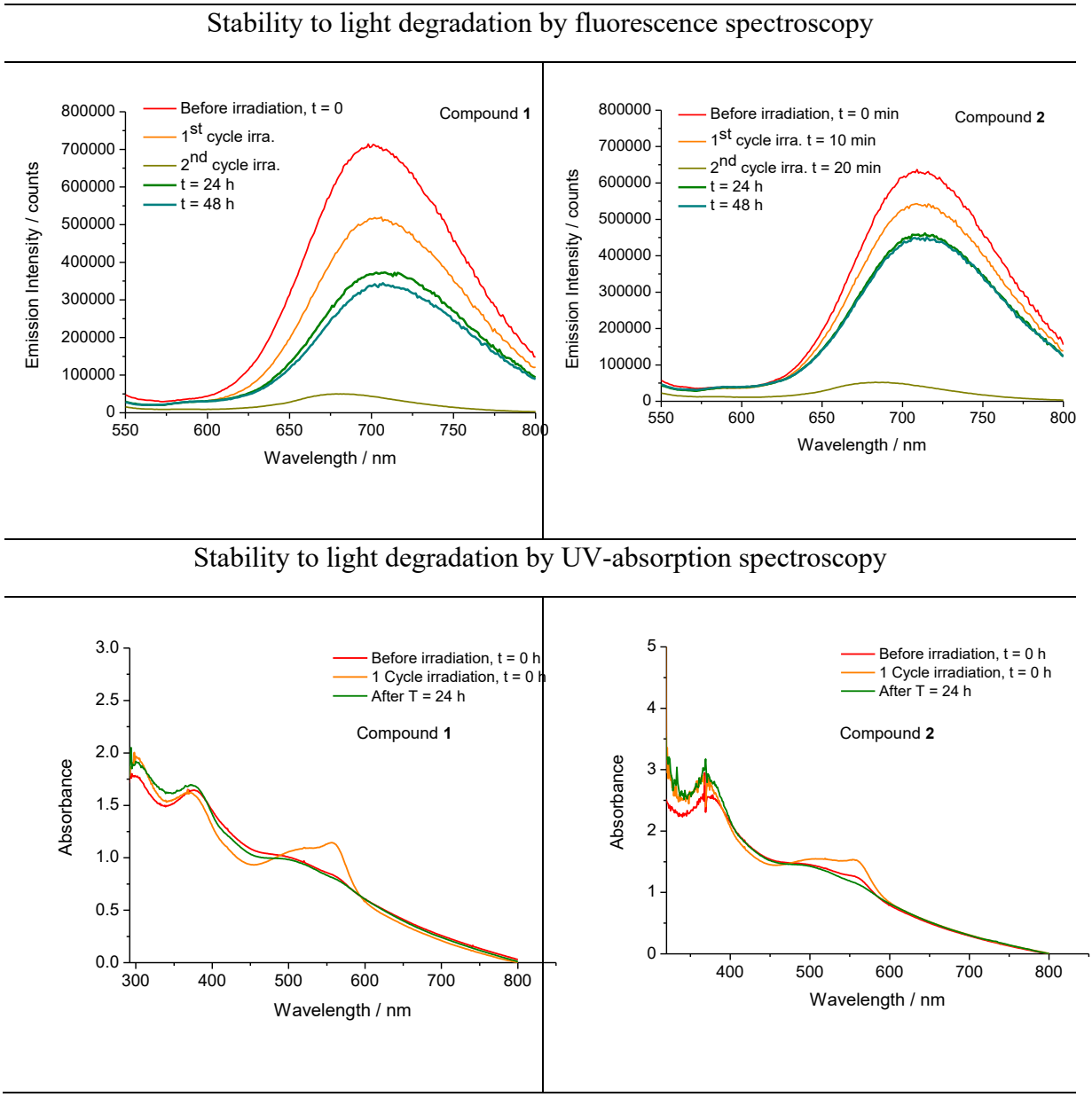


Figure S16. (Up) Emission spectra of complex **1** and **2** in DMEN (200µM) before irradiation at t=0, after two irradiation cycles (each cycle: 10 min at 470 nm) and after 24 and 48 h of the two cycles irradiation. (Down) Absorption spectra of complexes **1** and **2** in DMEN (200µM) before at t=0, after irradiation (10 min at 470 nm), and at 24 h.

Followed protocol for emission spectra: 1 mL of a solution of complexes **1** and **2** at 200 µM in DMEN was prepared from a 0.1 M DMSO stock solution of each complex. Then emission spectra were recorded before and after irradiation at 470 nm for 10 minutes. This process was repeated twice and then emission spectra was recorded at 24 and 48 h using a Jobin-Yvon-Horiba fluorolog FL-3-11 spectrometer using band pathways of 5 nm. *Followed protocol for absorption spectra:* 2 mL of a solution of complexes **1** and **2** at 200 µM in DMEN was prepared from a 0.1 M DMSO stock solution of each complex. Then three

absorption spectra were recorded (before irradiation, after irradiation at 470 nm for 10 minutes and after 24 h of irradiation). Experiment was performed un a Jasco Spectrophotometer using UV-cuvettes of 1cm path-length.

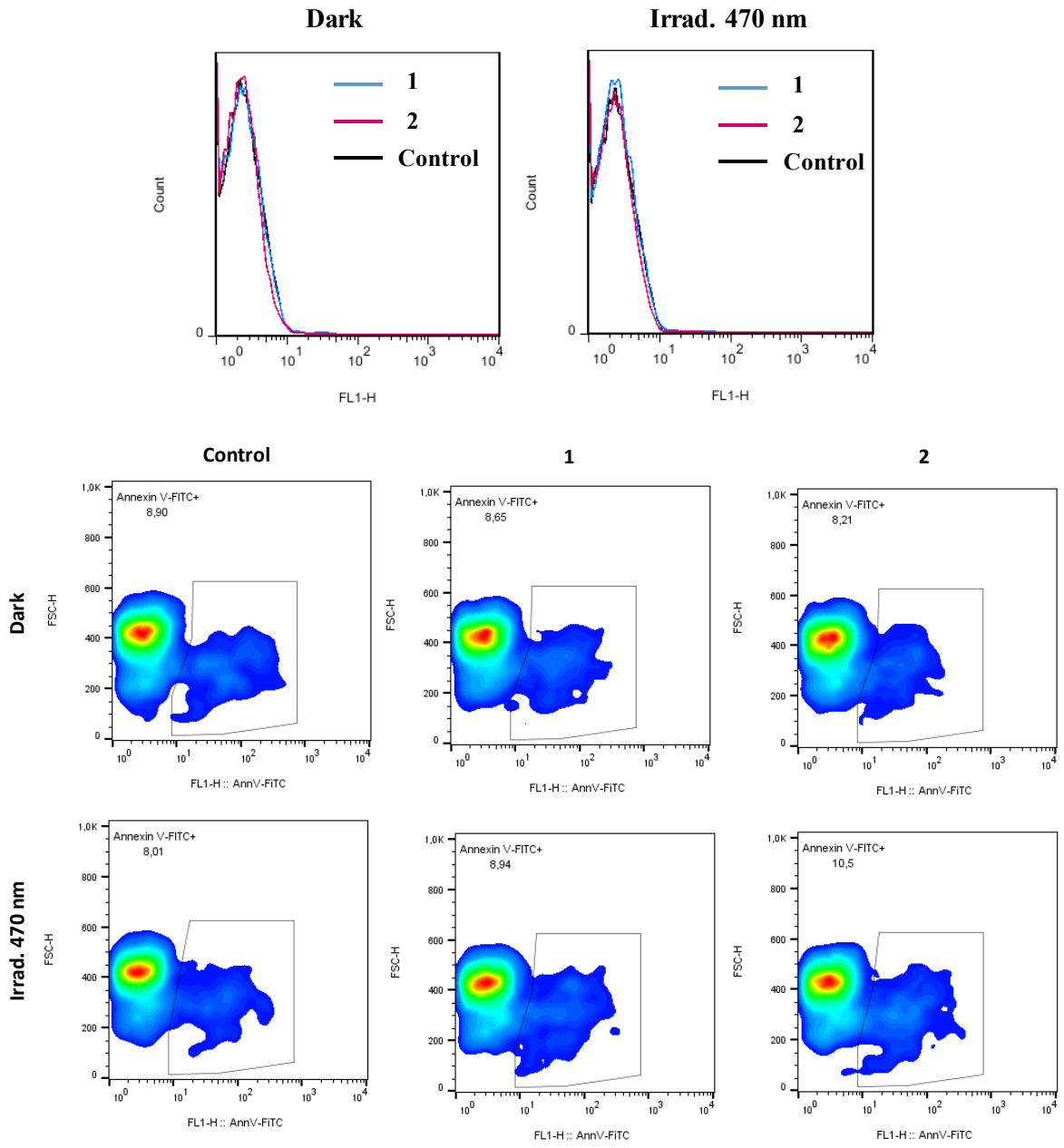


Figure S17. Cell death study at lymphocytes induced by complexes **1** and **2** (irradiation at 470 nm, 10 min, 100 μM) using Annexin V-FITC and related dot plots.

3 *IN VIVO* ASSAYS

3.1 Animals and housing.

All experiments followed the PI87/20 research procedures approved by the ethics committee for animal experiments of the University of Zaragoza. Animal care and use was carried out in accordance with the Spanish policy for animal protection RD53/2013 and the European Union directive 2010/63 for the protection of animals used for experimental and other scientific purposes. For the in vivo toxicology study, 8 sex-balanced mice (10-11 weeks) RjOrl:SWISS were used. 57 adult female BALB/c mice (5 weeks) were used in the in vivo oncological efficacy studies. All animals were purchased from Janvier Laboratories. Up to a maximum of 5 specimens were kept in 30 x 20 x 15 cm boxes, with access to osmotized and autoclaved water and irradiated food ad libitum (2914 Teklad Global 14% protein rodent maintenance diet). The room temperature was maintained at 23 ± 1 °C with a 12 h light cycle (starting at 8:00 a.m.).

3.2 *In vivo* toxicology assay.

Before testing the compounds in an in vivo efficacy study, we evaluated the in vivo toxicity of the compounds 1 and 2 that had shown very promising results in vitro. For such a purpose, an acute oral (PO) toxicity test was performed based on the OECD Test No. 425: Acute Oral Toxicity: Up-and-Down Procedure.[3] The highest dose tested was 175 mg/kg and the starting dose was 17,5 mg/kg, applying a slope of 2. The test was performed in mice of 10-11 weeks and toxicity was evaluated after a single administration.

The drugs were administered orally (PO) to conscious mice with a standard volume of 10 mL kg-1 body weight as a suspension in physiological saline (5% DMSO). Animals were monitored individually at least once during the first 30 min after injection, periodically during the first 24 h (with special attention to the first 4 h) and daily thereafter for a total of 14 days.

Health status of the animals was assessed using a modified scale based on the proposal by Morton and Griffiths.[4] General appearance (0-3), weight (0-2), spontaneous behavior (0-3), and provoked behavior (0-3) were scored. (Table 1).

Table S1. Score assessment scale.

PARAMETER	DESCRIPTION	SCORE
BODY CONDITION	10% ≤ Weight ≥ Day 0	0
	Weight ≤ 10%	1
	Weight ≤ 20%	2
APPEARANCE	NORMAL (bright eyes, shiny, well groomed hair coat)	0
	Unkempt hair coat	1
	Dull fur, presence eye or nasal secretions	2
	Hunching, piloerection	3
NATURAL BEHAVIOR	Normal (Active, interactive in environment)	0
	Pronounced decrease in activity or immobile	3
PROVOKED BEHAVIOR	Normal (Quickly moves away)	0
	Does not move or reacts with excessively exaggerated response	3
		TOTAL 11

Table S2. Time course and reversibility in onset of signs of toxicity.

Animal	Animal	Animal	Animal	Animal	Animal	Animal	Animal	Animal	Observations
1	1-B	17.5	0	0	1	0	O	O	No sign of toxicity
2	1-C	175	0	0	0	0	O	O	No sign of toxicity
3	1-C	175	0	0	0	0	O	O	No sign of toxicity
4	1-C	175	0	0	0	0	O	O	No sign of toxicity
5	2-B	17.5	0	0	0	0	O	O	No sign of toxicity
6	2-C	175	0	0	0	0	O	O	No sign of toxicity
7	2-C	175	0	0	0	0	O	O	No sign of toxicity
8	2-C	175	0	0	0	0	O	O	No sign of toxicity

* signs of toxicity reversibles

* signs of toxicity no reversibles

Table S3. Body weight & Body weight changes

Animal	Substance	Dose (mg/kg)	Weight 0 (g)	Weight 24h (g)	Weight 48h (g)	Weight 1w (g)	Weight 2w (g)	Δ 24h (%)	Δ 48h (%)	Δ 1w (%)	Δ 2w (%)
1	1-B	17.5	37	36.5	36.9	37.6	39	-1.35%	-0.27%	1.62%	5.41%
2	1-C	175	43.5	42.1	42.6	43.1	45.5	-3.22%	-2.07%	-0.92%	4.60%
3	1-C	175	30.2	30.9	31.7	34.5	34.8	2.32%	4.97%	14.24%	15.23%
4	1-C	175	32.9	33	33.1	29.9	33.5	0.30%	0.61%	-9.12%	1.82%
5	2-B	17.5	39.2	38.8	39.6	40.9	42	-1.02%	1.02%	4.34%	7.14%
6	2-C	175	39.9	38.5	39.7	40.4	40.2	-3.51%	-0.50%	1.25%	0.75%
7	2-C	175	32.2	30.7	30.8	34.4	34.8	-4.66%	-4.35%	6.83%	8.07%
8	2-C	175	34.8	33.7	34.9	32.6	34.6	-3.16%	0.29%	-6.32%	-0.57%

5-10% *10-15%* *15-20%* ***>20%***

3.3. *In vivo* oncological efficacy assays.

To confirm the in vivo chemotherapeutic activity of the compounds **1** and **2**, both of them were subjected to an efficacy assay in a syngeneic cancer mouse model with the 4T1-luc2 cell line (CRL-2539-LUC2™ / ATCC). Two trials were carried out: in the first trial, we performed the treatment early (day 1 post-implantation) and in the second trial we treated tumors at an advanced stage (from day 14 post-implantation).

3.3.1. First Trial – Early treatment.

4T1 mouse mammary tumor cells expressing luciferase was implanted subcutaneously in mice and visualized using optical imaging (IVIS Lumina XRMS, Perkinelmer) to monitor tumor growth non-invasively in a longitudinal study.

The study was carried out in 4 experimental groups: Control, compounds **1**, **2** and 5-Fluorouracil (SIGMA-Aldrich). Each experimental group consisted of 9 female mice of the BALB/cByJRj strain (Janvier Labs) with an age of 5 weeks and a mean weight of 19.26 g (±1,22 g).

On day 0, all animals (n=36) were implanted by subcutaneous administration in the lumbar region with $1x10^6$ tumor cls in a volume of 200 µl of phosphate buffered saline (PBS). On day 1, after optical imaging follow-up, the experimental groups were established. At that time, the mean radiance of each experimental group was set between 9,62 x 10^7 and 1,06 x 10^8 photon/sec/cm^2/sr. The beginning of treatment was on day 1 once the experimental groups were balanced.

The trial had a total duration of 11 days from the day of inoculation of the tumor cells. Optical imaging follow-up, weight change and treatment were performed on days 1, 5, 7, and

11. Treatment was performed orally with a sterile metal feeding tube 22 ga x 25 mm (FTSS-22S-25, Instech) after image acquisition at a concentration of 1 mg/kg. Drugs were administered orally (PO) to conscious mice with a standard volume of 10 mL kg^{-1} body weight as a suspension in physiological saline (5% DMSO). Five minutes before optical imaging acquisition, a volume of 10 mL kg^{-1} body weight of a luciferin (D-Luciferin, potassium salt, DELTACLON S.L) solution [15 mg / mL] was administered intraperitoneally to each mouse.

Table S4. Averaged BLI data for each experimental group over time.

Group	Day 1	Day 5	Day 7	Day 11
Control	9.77E+07	1.70E+09	5.09E+09	2.73E+10
1	1.05E+08	6.12E+08	8.34E+08	1.28E+09
2	9.91E+07	8.03E+08	8.28E+08	6.02E+09

3.3.2. Second Trial – Delayed treatment.

4T1-luc2 cells were implanted subcutaneously into mice and tumor growth was monitored non-invasively in a longitudinal study through caliper measurements.

The study was carried out in 2 experimental groups: Control and Treated (compound **1**). Each experimental group consisted of 10 and 11 (respectively) BALB/cByJRj female mice with an age of 5 weeks and an average weight of 17.9 g (±1,054 g).

On day 0, all animals (21) were implanted with $1x10^6$ 4T1-luc2 cells in the lumbar region by subcutaneous administration. The trial had a total duration of 32 days from the day of inoculation of the tumor cells. Once the tumors were around 200 mm^3 (day 11), they were measured with a caliper and each of the animals was assigned to one of the two experimental groups in a balanced manner. Caliper follow-up, weight change and treatment were performed on days 11, 14, 18, 21, 25 and 32. Treatment administration was performed as previously described for trial 1. To assess tumor growth, the length and width of the tumor was measured using a caliper; The mean tumor volume was calculated using the following formula: Tumor volume = ½ (length × $width^2$).[5]

Table S5. Averaged caliper data for each experimental group over time.

Group	Day 11	Day 14	Day 18	Day 21	Day 25	Day 32
Control	199.07	348.79	533.41	832.15	1088.97	1742.76
1	214.63	291.98	327.82	383.21	478.75	805.06

Table S6. Trial 2: Mice with ulcerated tumors and the specific euthanasia day.

Mice	GROUP	Euthanasia	Ulcerated Day 32	Ulcerated Day 39
1	Treated	Day-32	YES	DEAD
2	Treated	Day-39	NO	NO
3	Treated	Day-32	YES	DEAD
4	Treated	Day-39	NO	NO
5	Treated	Day-39	NO	NO
6	Treated	Day-39	NO	YES
7	Treated	Day-39	NO	NO
8	Treated	Day-39	NO	NO
9	Treated	Day-39	NO	NO
10	Treated	Day-32	YES	DEAD
11	Treated	Day-32	YES	DEAD
1	Control	Day-32	YES	DEAD
2	Control	Day-32	YES	DEAD
3	Control	Day-32	YES	DEAD
4	Control	Day-32	YES	DEAD
5	Control	17-Jan	NO	NO
6	Control	17-Jan	NO	NO
7	Control	Day-32	YES	DEAD
8	Control	Day-32	YES	DEAD
9	Control	Day-32	YES	DEAD
10	Control	Day-32	YES	DEAD

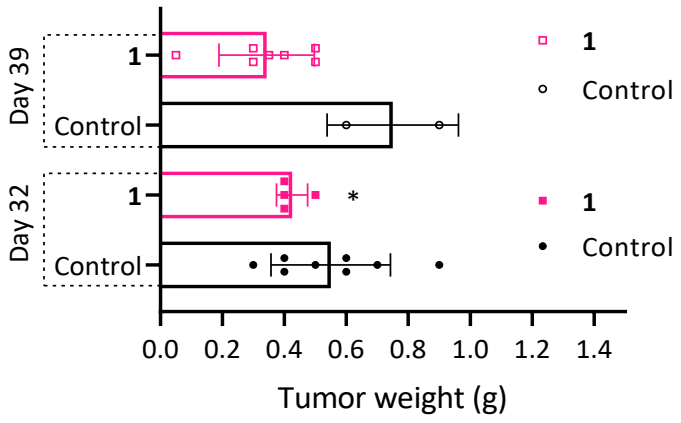


Figure S18. Tumor weights for control group and **1** after euthanasia at days 32 and 39.

3.4.Tumor weight prediction

To compare all mice, a tumor weight predictive model was developed. Assuming that caliper and tumor weight are directly related, day 39 tumor weight can be related with day 39 caliper. Then, using the unique tumor growth model for each mouse, it is possible to predict the caliper at day 32 and, thus, interpolate an approximation of the tumor weight at day 32 (Figure S13).

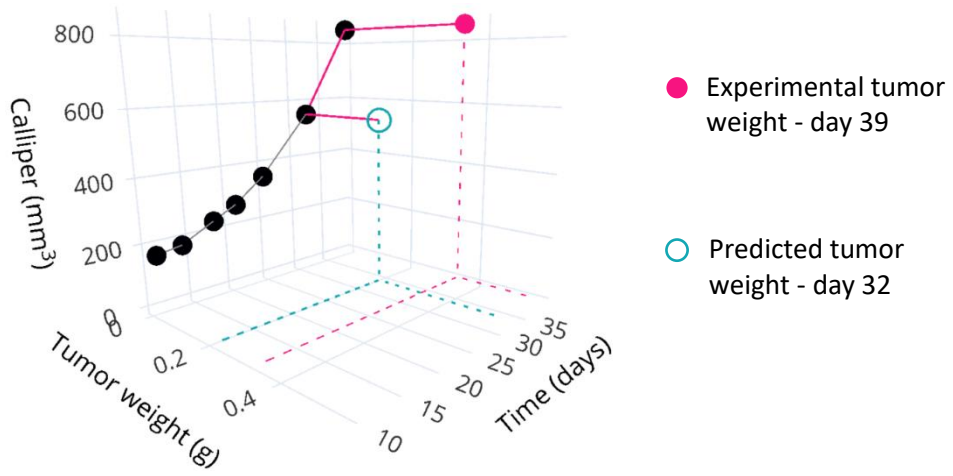


Figure S19. Tumor weight-in-terms-of-caliper predicting model example.

The precision of the model could be validated calculating the error produced by the caliper model, as the day 32 and day 39 caliper values were measured (Table S5).

Table S5. Example of the tumor weight interpolation methodology for a mice within the treated group.

	DAY 39	Day 32
Caliper (mm^3)	898.3	638.5
Predicted caliper (mm^3)	883.4	666.3
Tumor weight (g)	0.40	-
Predicted tumor weight (g)	-	0.30

At day 39, the difference between the measured and the predicted caliper is 1.2%. At day 32, the difference between the measured and the predicted caliper is 2.2%.

3.5. Tumor growth models: Methodologies

Due to *intra*-group variability, a tumor growth model was developed to evaluate the degree of tumor-affecting that treated experimental groups (**1** and **2**) present against the control group. BLI and caliper data were fitted to equations 1 and S1 using Excel's exponential regression analysis.

$$V_x(t) = V_0 e^{rt}$$

Equation S1. Tumoral evolution model over time in terms of caliper where V_x is the tumor volume at day x, V_0 is the tumor volume at day 0, r is the growth rate constant, and t is the time.

Assuming an exponential growth rate for the caliper evolution, r is calculated after a mathematic transformation of equations 1 and S1 (Eq. 2 and Eq. S2).

$$r = \frac{\ln (V_x/V_0)}{t_x - t_0}$$

Equation S2. Tumor growth constant r calculus from caliper data over time.

Then, r_{calc} (obtained from Eq. 2 and Eq. S2) is obtained as an average of all the r constants calculated from Equations 2 and S2 (BLI model and caliper model, respectively).

r_{calc} is compared to the fitted one (r_{exp}, obtained from Eq. 1 and Eq. S1) to evaluate the precision of the fitting model (Figure S14).

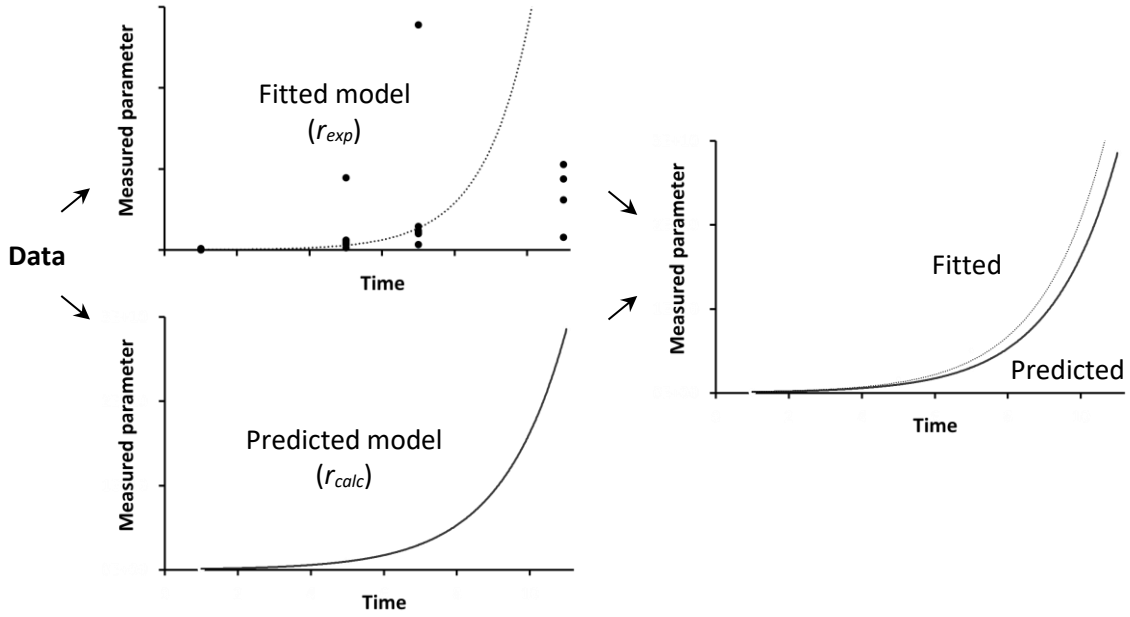


Figure S20. Developed methodology for the evaluation of the applicability of the tumor growth model to the experimental data.

This methodology confirmed the exponential tendency that tumor growth shows in both studies (BLI and caliper). Comparison of the averaged *r* constants between experimental groups quantified the rate change of the tumor growth, and hence, the efficacy of **1** and **2**.

3.5.1. Data for *r* calculation: *BLI experiment (early treatment):*

Fitted tumor growth functions for BLI experiment and the R^2 values (Equations S3-S5):

$$BLI(t) = 7x10^7 e^{0.5626rt}$$

$$R^2 = 0.9942$$

Equation S3. Fitted exponential equation for the control group in the BLI experiment and its R^2 value.

For the control group, r_{exp} is 0.5626 days^{-1}.

$$BLI(t) = 9x10^7 e^{0.2577rt}$$

$$R^2 = 0.9778$$

Equation S4. Fitted exponential equation for **1** in the BLI experiment and its R^2 value.

For **1**, r_{exp} is 0.2577 days^{-1}.

$$BLI(t) = 7x10^7 e^{0.3954rt}$$
$$R^2 = 0.9903$$

Equation S5. Fitted exponential equation for **2** in the BLI experiment and its R^2 value.

For **2**, r_{exp} is 0.3954 days^{-1}.

Calculated *r* constants from Equation 2 for each experimental group in the BLI experiment (early treatment):

Table S7. Calculated *r* constants for the control group in the BLI experiment.

Time (days)				
1	5	7	11	
	0.71	0.66	0.56	1
0.71		0.55	0.46	5
0.66	0.55		0.42	7
0.56	0.46	0.42		11

Time (days)

The averaged r_{calc} for the control group is **0.56±0.03**.

Table S8. Calculated *r* constants for **1** in the BLI experiment.

Time (days)				
1	5	7	11	
	0.44	0.35	0.25	1
0.44		0.15	0.12	5
0.35	0.15		0.11	7
0.25	0.12	0.11		11

Time (days)

The averaged r_{calc} for the control group is **0.24±0.04**.

Table S9. Calculated *r* constants for **2** in the BLI experiment.

Time (days)				
1	5	7	11	
	0.52	0.35	0.41	1
0.52		0.01	0.34	5
0.35	0.01		0.50	7
0.41	0.34	0.50		11

Time (days)

The averaged r_{calc} for the control group is **0.36±0.05**.

3.5.2. Data for *r* calculation: *Caliper experiment (delayed treatment)*

Fitted tumor growth functions for caliper experiment and the R^2 values (Equations S6 and S7):

$$V(t) = 80.65e^{0.1014rt}$$

$$R^2 = 0.9599$$

Equation S5. Fitted exponential equation for the control group in the caliper experiment and its R^2 value.

For the control group, r_{exp} is 0.1014 days^{-1}.

$$V(t) = 115.53e^{0.0591rt}$$

$$R^2 = 0.9864$$

Equation S6. Fitted exponential equation for **1** in the caliper experiment and its R^2 value.

For **1**, r_{exp} is 0.0591 days^{-1}.

Calculated *r* constants from Equation S2 for each experimental group in the caliper experiment (delay treatment):

Table S10. Calculated *r* constants for the control group in the caliper experiment.

Time (days)							
11	14	18	21	25	32		
	0.19	0.14	0.14	0.12	0.10	11	
0.19		0.11	0.12	0.10	0.09	14	
0.14	0.11		0.15	0.10	0.08	18	Time
0.14	0.12	0.15		0.07	0.07	21	(days)
0.12	0.10	0.10	0.07		0.07	25	
0.10	0.09	0.08	0.07	0.07		32	

The averaged r_{calc} for the control group is **0.11±0.02**.

Table S11. Calculated *r* constants for **1** in the caliper experiment.

Time (days)							
11	14	18	21	25	32		
	0.10	0.06	0.06	0.06	0.06	11	
0.10		0.03	0.04	0.04	0.06	14	
0.06	0.03		0.05	0.05	0.06	18	Time
0.06	0.04	0.05		0.06	0.07	21	(days)
0.06	0.04	0.05	0.06		0.07	25	
0.06	0.06	0.06	0.07	0.07		32	

The averaged r_{calc} for the control group is **0.06±0.01**.

3.6. Statistical analysis: Methodologies

The normality of the data in each experimental group, including bioluminescence (early treatment), caliper measurements, and tumor weight (delayed treatment), was assessed by conducting a Shapiro-Wilk test. The results revealed that most data did not conform to a normal distribution. Consequently, statistical comparisons were performed using non-

parametric methods. The Mann-Whitney U test was employed to assess the equality of the various populations. The test was applied in its two-tailed version with a confidence level of 95%. All the statistical comparisons were performed using GraphPad Prism 9.

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