Supplementary Information for

Cell viability imaging in tumour spheroids via DNA binding of a ruthenium (II) light-switch complex

Vadde Ramu*[a], Lukas S. Wijaya,[b] Nataliia Beztsinna,[a] Corjan Van de Griend,[a] Bob van de Water,[b] and Sylvestre Bonnet *[a] Sylvia E. Le Dévédec*[b]

a *Leiden Institute of Chemistry, Leiden University, P.O Box 9502, 2300 RA Leiden, The Netherlands.*

b *Leiden Academic Center for Drug Research, Leiden University, P.O Box 9502, 2300 RA Leiden, The Netherlands.*

Correspondence to ramuvadde1@gmail.com bonnet@chem.leidenuniv.nl, s.e.ledevedec@lacdr.leidenuniv.nl

Table of Contents

Materials and methods

Human cancer cell lines (HepG2, human hepatocarcinoma; U87, human glioblastoma) were distributed by the European Collection of Cell Cultures (ECACC) and purchased through Sigma Aldrich. Dulbecco's Minimal Essential Medium (DMEM, high glucose, without glutamine), 200 mM Glutamine-S (GM), trichloroacetic acid (TCA), glacial acetic acid, Sulforhodamine B (SRB), tris(hydroxylmethyl)aminomethane (tris base), and *cis*-diamineplatinum(II) dichloride (cisplatin) and staurosporine were purchased from Sigma Aldrich. (2*R*,3*R*,4*R*,5*R*)-hexan-1,2,3,4,5,6-hexol (D-mannitol) was purchased from Santa Cruz Biotechnology via Bio-Connect. Fetal calf serum (FCS) was purchased from Hyclone. Penicillin and streptomycin were purchased from Duchefa and were diluted to a 100 mg/mL penicillin/streptomycin solution (P/S). Trypsin and Opti-MEM® (without phenol red) were purchased from Gibco® Life Technologies. Trypan blue (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate dibasic solution) was purchased from Bio-Rad. Plastic disposable flasks and 96-well plates were from Sarstedt. Cells were counted using a Bio-Rad TC10 automated cell counter with Bio-Rad Cell Counting Slides. 96-well plates used for confocal imaging were either CellStar µ-clear (ref 655090) or ScreenStar µ-clear (ref 655866) from Greiner Bio One. 96-well spheroid microplates (ref 4515) were purchased from Corning via Sigma Aldrich. UV-vis measurements for analysis of 96-well plates were performed on a M1000 Tecan Reader. All fluorescent dyes, unless specifically mentioned, were purchased from Thermo Fisher Scientific.

Cell culture

HepG2, A549, and U87 Cells were thawed and at least passaged twice before starting cytotoxicity and uptake experiments. Each cell line was cultured in Dulbecco's Modified Eagle Medium with phenol red, supplemented with 10.0% v/v fetal calf serum (FCS), 0.2% v/v penicillin/streptomycin (P/S), and 0.9% v/v Glutamine-S (GM). Cells were cultured in either 25 cm^2 or 75 cm^2 flasks and split at 70-80% confluence. The flasks were incubated in a normoxic PHCbi incubator at 37 °C at 5.0% CO_2 in O_2/CO_2 incubator, MCO-170M). The medium was refreshed twice a week. Cells used in all biological experiments were cultured for not more than maximum of eight weeks.

Compound 1 cellular uptake in 2D cultures

Cells were seeded in either CellStar µ-clear (ref 655090) or ScreenStar µ-clear (ref 655866) 96-well plates and allowed to attach overnight. When needed, cells were pre-treated with cytotoxins (cisplatin 25 or 50 µM; staurosporine 5 or 10 µM) for 24 h. Then the media was refreshed, and appropriate fluorescence staining was added to each well. The nuclei were stained with Hoechst formulation from ReadyProbes following the manufacturer's protocol (NucBlue, 2 drops/mL, 10 min incubation), necrotic cells with Propidium Iodide formulation from ReadyProbes following the manufacturer's protocol (2 drops/mL, 20 min incubation), apoptotic cells with Annexin V Pacific Blue conjugate in accordance with manufacturer protocol (Thermo Fisher Scientific). $[Ru(bpy)_2(dppz)](PF_6)_2$ (**1**) was prepared as

concentrated stock solution in DMSO (1 mM) and then diluted with medium (DMEM complete) to a final concentration of 25 or 50 µM in each well.

Cellular uptake of compound 1 in 3D tumor spheroids

Cells were seeded in 96-well spheroid microplates (Corning, ref 4515) at required cell densities (from 300 to 4000 cells/well depending on the experiment). After 3-4 days in culture spheroids were fully formed and then subjected to either 24 h pre-treatment with cytotoxins (cisplatin 50 or 100 µM; staurosporine 5 or 10 µM) or directly compound **1** uptake experiments. Colocalization studies were performed using Hoechst (NucBlue, 2 drops/mL, 10 min incubation) and Propidium (20µM) iodide dyes.

Image acquisition

The imaging was performed on a Nikon Eclipse Ti2 $C2^{+}$ confocal microscope (Nikon, Amsterdam, The Netherlands) and this system included an automated XY-stage, an integrated Perfect Focus System (PFS), and 408 and 561 lasers. The system was controlled by Nikon's NIS software. All images were acquired using a 20x objective with 0.7 NA, at a resolution of 512×512 pixels. For the imaging of the 2D monolayers in 96 flat bottom plates, 4 positions per well were acquired every 30 minutes for a total of 16 hours. For the imaging of the 3D spheroids in U bottom plates, 15 focal planes were imaged every 15 µm for at least 48 hours in each well containing one spheroid. Hoechst was imaged at $\lambda_{ex/em}$ 405/450 nm and **1** or Propidium Iodide at $\lambda_{ex/em}$ 488/650 nm.

Image Analysis

2D image analysis: ROIs of the same area were manually added to the raw images where nuclei displaying a cell death phenotype were selected. Next, the total intensity for both channels in the ROIs [Hoechst ($\lambda_{ex/em}$ = 350/461) and Cy3 ($\lambda_{ex/em}$ = 555/569)] were quantified using NIS analysis software. The mean total intensity for about 10 nuclei was plotted vs. time.

3D image analysis: The images from the imaging experiments were stored in. nd2 format and processed stepwise. The z-stack images were projected based on the highest intensity using NIS element analysis software. The projected images were then extracted to obtain separated images from every channel in each condition (see supplemental figure 2). These images were then applied to the high-content imaging pipeline for quantification. The quantitative image analysis was performed with ImageJ version 1.52p and CellProfiler version 2.2.0.[1] The nuclei per image were segmented by watershed masked algorithm on ImageJ as the seeds and thereafter processed with an in-house developed CellProfiler module.[2] We applied nuclear identification from the segmented nuclear images to quantify the number of nuclei. Error bars represent SD for approximately 5 spheroids per condition. The quantification of both signals in ~10 nuclei was performed: In cells co-stained with PI the average signal intensity of Hoechst in the nuclei was found to be stable while the PI signal increased vs. time. Further, we also quantify the total nuclear area. The PI or **1** area was also quantified to reflect the magnitude of the cell death. The response of the cell death was then expressed as the ratio of the PI/**1** area to the nuclear area. The results were stored as HDF5 files. Data analysis, quality control, and graphics were performed using the in-house developed R package h5CellProfiler[2]

Cell viability assay in 3D spheroids

Cell viability assessment studies were performed in both HepG2 and U87 spheroids using Celltiter Glo 3D assay as per the literature reported protocols. HepG2, U87 cells were cultured, trypsinezed and counted using BioRad cell counting machine with tryphon blue dye. Each cell type was seeded with seeding density of 300 cells/well on day 0, incubated at 37 °C, 7% CO_2 for 3 days to form spheroids.

On the day 4 after seeding the spheroid size and morphology were assessed by phase contrast imaging with Evos FL Auto 2 imaging system (Thermo Fisher Scientific) at 4x magnification. The average spheroid diameter was quantified by manually measuring its diameter in x and y axis using Fiji v 2.0.0 software (open-source software platform from GitHub). Cisplatin positive control solution (50µM) was prepared from a stock solution based on clinical formulation (3.3 mM cisplatin, 55 mM mannitol, 154 mM NaCl). To overcome the poor water solubility of compound **1**, sterilized dimethyl sulfoxide (DMSO) was used to dissolve the complexes in such amounts that the maximum v/v% of DMSO per well did not exceed 0.5% v/v%.

A complete 3D (photo) cytotoxicity experiment lasted 7 days: cells were seeded at t = day 0, treated at t = day 4, and viability assessed at t = day 7. 96-well spheroid microplates were seeded with the correct number of cells in 200 µL DMEM complete per well, on day 4 100 µL per well was pipetted out and replaced with the 100 µL per well of compounds diluted in Opti-MEM complete. In the remaining outer wells 200 µL of Opti-MEM complete was pipetted. On day 4 the spheroids were treated with compound **1** with varied concentrations ranging from 0 to 100 µM followed by incubation for 48 h. The endpoint viability of spheroids was assessed with Celltiter Glo 3D assay from Promega in accordance with manufacturer instructions. Briefly, both the Celltiter Glo 3D reagent and the plates with spheroids were left to equilibrate at room temperature for 30 min. Then 100 µL of Celltiter Glo 3D reagent were pipetted into each well and the plates were shaken on a plate shaker for 5 min to disintegrate the spheroids and then further incubated for 25 minutes at room temperature. To determine the spheroids viability the total luminescence (integration time 20 ms) was measured using a M1000 Tecan Reader. The luminescence data per compound per concentration were averaged over nine spheroids from two independent biological replicates in Excel and made suitable for use in GraphPad Prism. Relative cell populations were derived from the average of the untreated controls (n_t = 6 per replicate).

Cryosectionning of 3D spheroids

HepG2 and U87 spheroids were prepared, incubated with **1**, PI or Hoechst as described in the Cellular uptake section above and imaged with a Nikon Eclipse Ti2 C2$^+$ confocal microscope (see Image acquisition section). Next, the spheroids harvested one by one, embedded, and frozen in Tissue-Tek® O.C.T solution (Sakura Finetek USA, Torrance, CA, USA). In total 3 to 6 spheroids from each treatment group were embedded per O.C.T. block. Five-micrometre cryo-sections of the blocks were generated using Leica CM3050S cryostat. The sections were deposited on glass slides and imaged using the same microscope system as described above.

Table S1. Cytotoxicity values for complex **1** and cisplatin was assessed by Sulforhodamine B assay in HepG2 and U87 cells.

Compound	Cell type	EC_{50} (µM)	+Error	-Error
1	HepG2	73.8	118	45.3
	U87	207	150	85.7
Cisplatin	HepG2	2.3	0.9	0.7
	U87	7.1	3.9	2.7

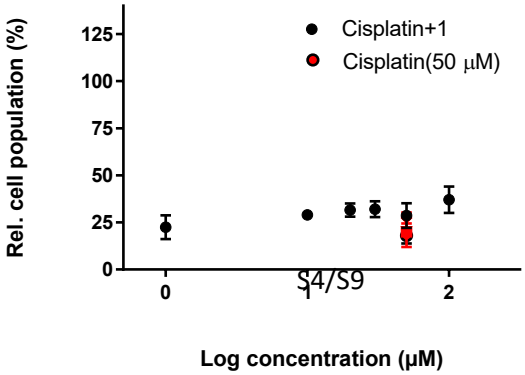

Figure S1. Resulting cytotoxicity data for testing synergy between cisplatin (50 μM) and **1** (50 μM) in U87 cells.

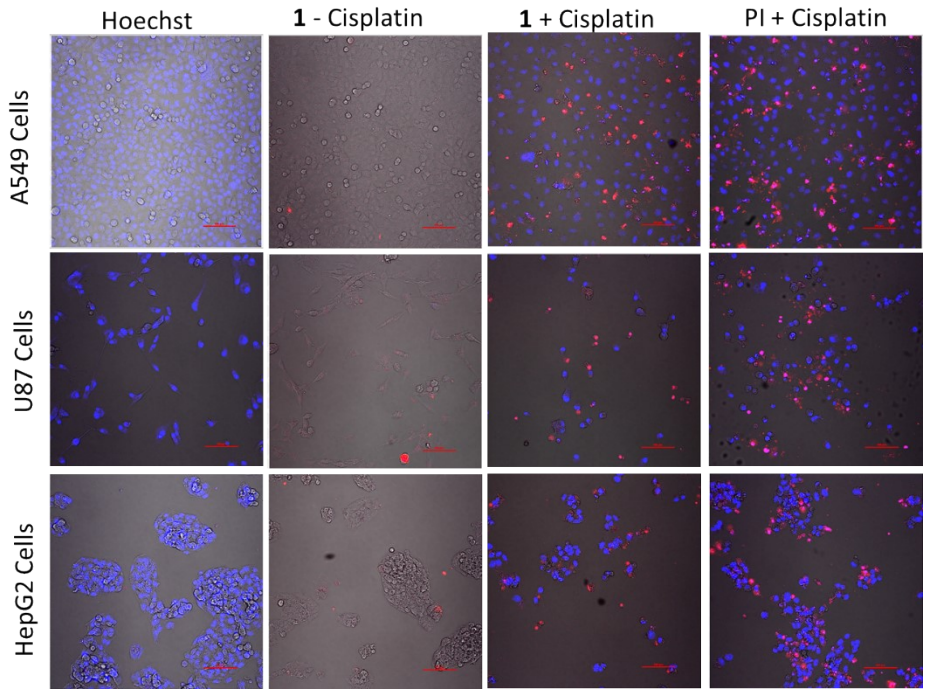

Figure S2. Confocal live cell images of A549, U87 and HepG2 cells treated with Hoechst, **1** (50μM) and PI (20μM) treated with or without Cisplatin (50μ) for 16 h.

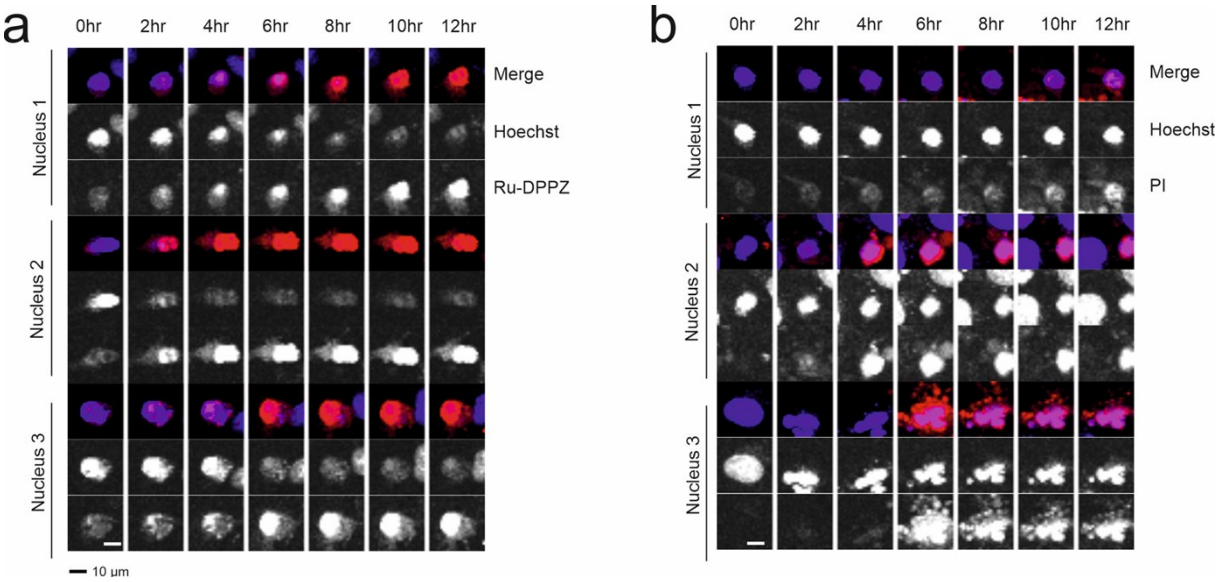

Figure S3. Time-dependent displacement of Hoechst by **1** or propidium iodide (PI) in dying A549 cells treated with cisplatin (50 µM) for 16 h. Cells stained with either Hoechst and **1**(a) or Hoechst and PI (20µM) (b). Scale bar is 10 µm.

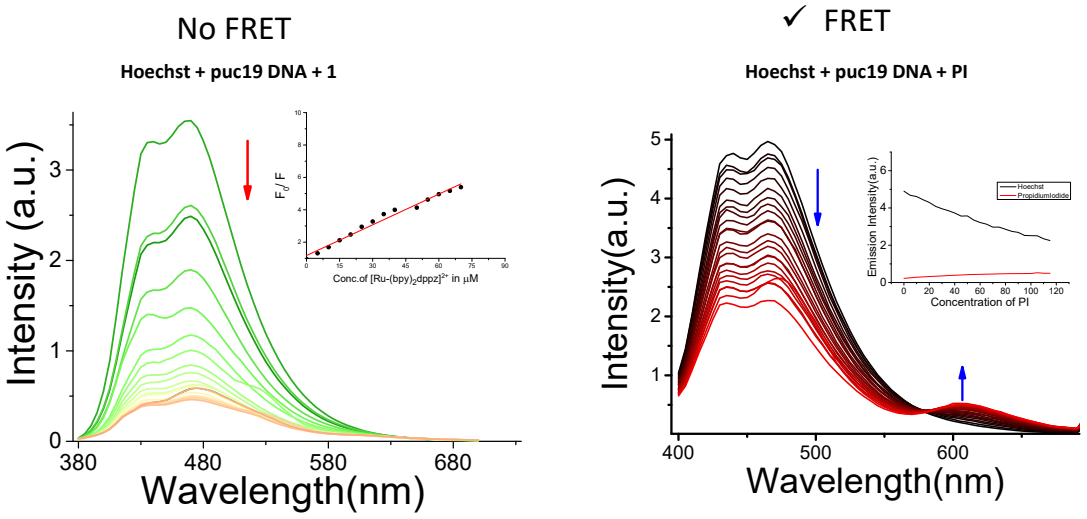

Figure S4. Emission spectra of Hoechst when combined with pUC19 DNA in PBS buffer and quenched with varying concentrations of (ranging from 0 to 80 µM) either **1** (left side graph) (a) or (b) PI (right side graph). The inset displays the Stern-Volmer plot at the wavelength λ_{max} 480 nm for **1** and λ_{max} 480, 610 nm for PI.

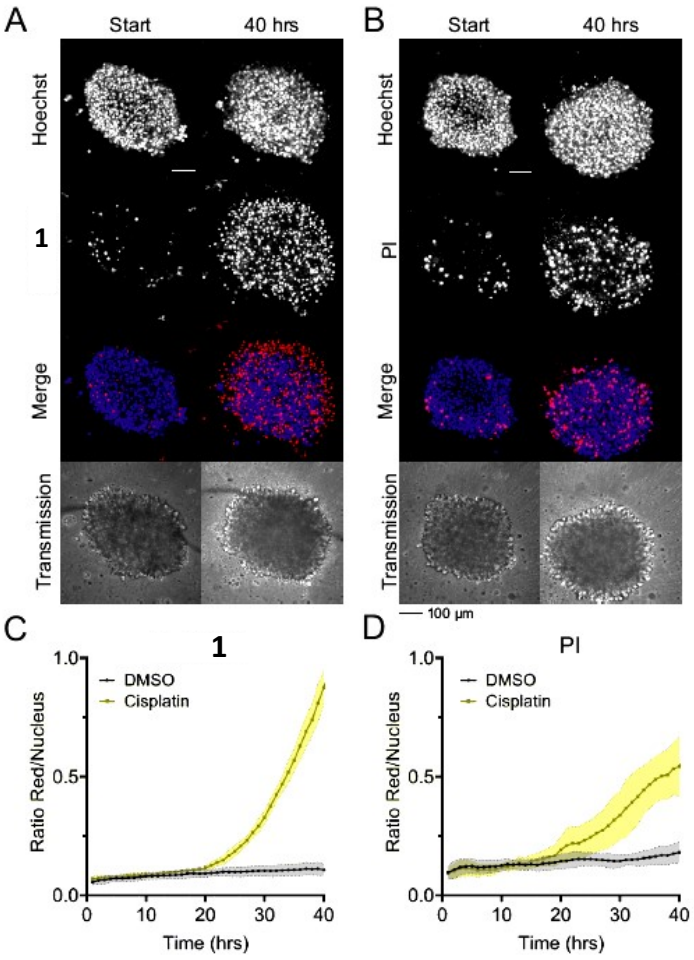

Figure S5. Live imaging of cell death in U87 tumor spheroids exposed to cisplatin (50 µM) using either **1** (50 µM) or PI as dyes for dying cells (another replicate). Example images of tumor spheroids exposed to cisplatin (50 µM) at the start and end of the time-lapse stained with either Hoechst or **1** (50 µM) (A) or Hoechst and Propidium Iodide (B). Scale bar is 100 µm. Quantification of the **1** signal ratio over the nuclear signal for either **1** staining (C) or PI staining (D). Error bars indicate standard deviation for about 5 spheroids per condition.

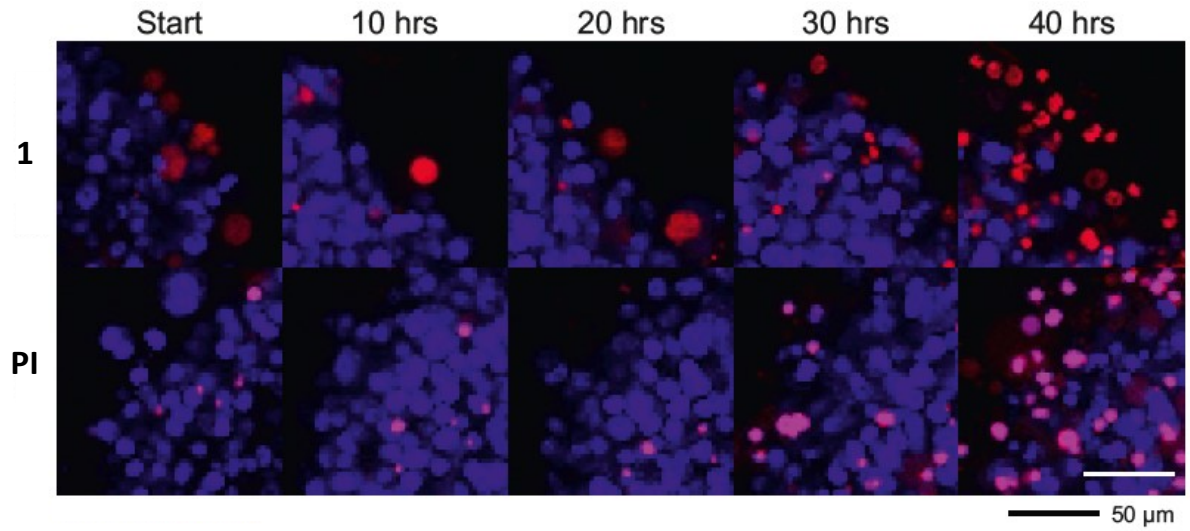

Figure S6. Time lapses confocal images of the selected regions of U87 tumor spheroids captured from 0-40 h revealed distinct co-localization patterns with Hoechst being displaced by compound **1** while the staining is maintained with PI. Scale bare 50 µm.

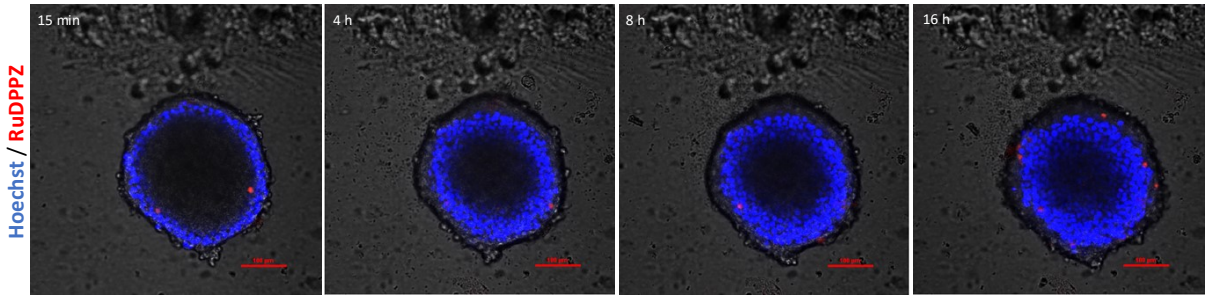

Figure S7. Healthy HepG2 spheroids stained with Hoechst and **1** in the absence of cisplatin displayed no staining by **1** as there is no dead cells. Scale bar 100 µm.

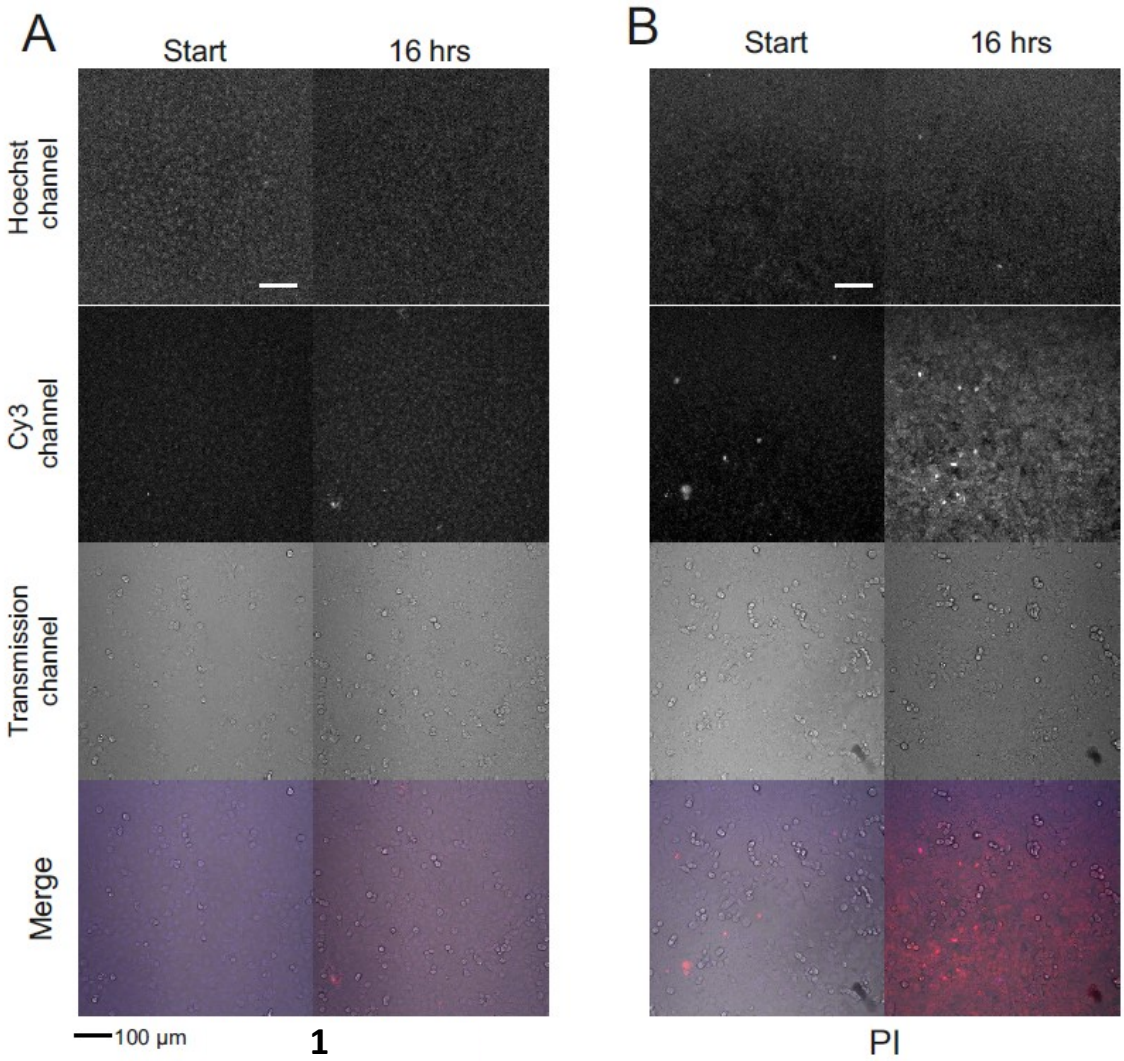

Figure S8. Background signal of **1** (50 µM) (A) and PI (B) in the A549 cells in all three detection channels used for the time-lapse imaging. Cells were not exposed to cisplatin nor stained with Hoechst. Scale bar is 100 µm. Imaging started at 0 to 16 hours.

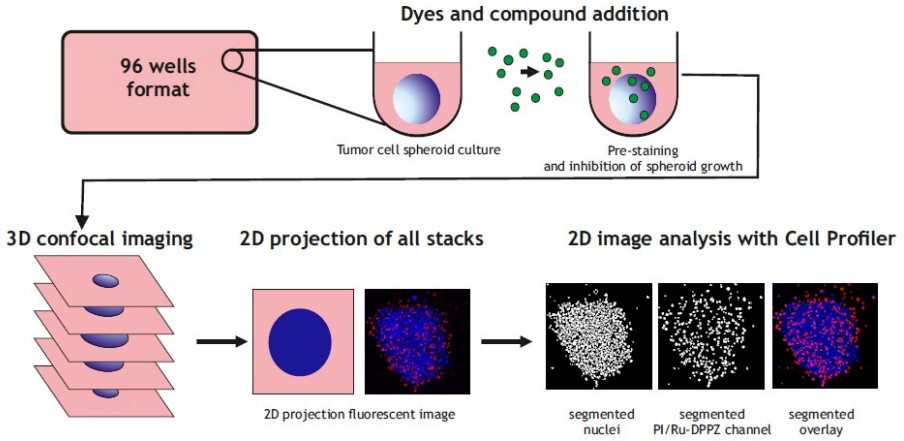

Figure S9. Overview of the image analysis workflow used to quantify cell death over the time in the 3D spheroids.

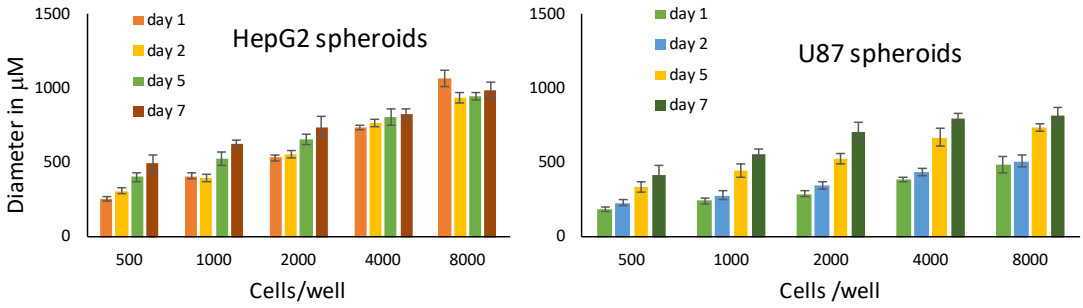

Figure S10. Spheroid growth bars represents the growth monitored from day 1 to day 7. Opti-MEM media refreshed every second days.

References

1. L. Kamentsky, T. R. Jones, A. Fraser, M. A. Bray, D. J. Logan, K. L. Madden, V. Ljosa, C. Rueden, K. W. Eliceiri and A. E. Carpenter, *Bioinformatics (Oxford, England)*, 2011, **27**, 1179-1180.
2. S. Wink, G. A. Burger, S. E. L. Dévédec, J. B. Beltman and B. v. d. Water, 2022, DOI: 10.1101/2022.10.06.511212 %J bioRxiv, 1-16.