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Fluorescent Antitumor Titanium(IV) Salen Complexes for Cell Imaging

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Electronic Supplementary Information

Materials and Methods

Ligands **1a** and **2a** were synthesized according to known procedure[1] starting from the commercially available substituted salicylaldehyde and diamine. All ligands were dried at ~50 °C under vacuum for 12 h before complexation. 4-(Diethylamino)salicylaldehyde (99%) was purchased from Alfa Aesar; diaminomaleonitrile (98%) was purchased from Acros Organics; potassium tetrachloroplatinate(II) was purchased from Strem Chemicals; titanium(IV) tetraisopropoxide (97%) and *o*-phenylenediamine (99.5%) were purchased from Sigma Aldrich and all used without further purification. $Ti(OArMe_2)_4$ was synthesized as previously described.[2] **2a-Pt** was synthesized according to known procedure.[3] All solvents used for procedures requiring an inert atmosphere were purchased anhydrous or dried over alumina columns on M.Braun SPS-800 solvent purification system. All experiments requiring dry atmosphere performed in an M. Braun dry-box under nitrogen atmosphere. NMR data were recorded using AMX-400 or AMX-500 MHz Bruker spectrometer. X-ray diffraction data were obtained with Bruker Smart Apex diffractometer, running the SMART software package. After collection, the raw data frames were integrated by the SAINT software package. The structures were solved and refined using SHELXTL software package. Elemental analyses performed in the

microanalytical laboratory at the Hebrew University of Jerusalem. Accurate-Mass QTOF LC/MSMS measurements were carried on an Agilent Technologies 6520 instrument. The absorbance was measured using UV-Vis spectrophotometer Genesys 10S, Thermo Scientific; VISIONlite program. The emission was measured using spectrofluorophotometer RF-1501, Shimadzu; Panorama Fluorescence program. Photoluminescence quantum yields (PL-QY) were measured using Hamamatsu absolute PL-QY spectrometer C11347.

Cell Growth Inhibition Studies

Cytotoxicity was measured on HT-29 human colon colorectal adenocarcinoma cells and HeLa human cervix adenocarcinoma cells obtained from ATCC Inc. The Methylthiazolyldiphenyltetrazolium bromide (MTT) assay was employed as previously described,[4] starting from $0.6x10^6$ cells in medium (contains: 1% penicillin antibiotics; 1% L-glutamine; 10% fetal bovine serum (FBS) and 88% medium RPMI-1640 for HT-29 or 88% medium DMEM for HeLa, all purchased from Biological Industries and Sigma Aldrich) were seeded into a 96-well plate and allowed to attach for a day under incubation at 37 °C in 5% CO_2 atmosphere. The cells were consequently treated with the reagent tested at 10 different concentrations. Solution of reagent was prepared by dissolving 8 mg of the reagent in 200 μL of DMSO and diluting this solution to give final concentration in medium of up to 200 mg/L in 0.5% DMSO. After a standard of 3 days incubation at 37 °C in 5% CO_2 atmosphere, MTT (0.1 mg in 20 μL) was added and the cells were incubated for additional 3 h. The MTT solution was then removed, and the cells were dissolved in 200 μL isopropanol. The absorbance at 550 nm was measured by a microplate reader (Spark 10M, Tecan). Each experiment was repeated at least 3 × 3 times, namely, three repeats of the experiment per plate, all repeated at least 3 times on different days (9 repeats altogether). Relative IC_{50} values were determined by a nonlinear regression of a variable slope (four parameters) model, and are presented as mean ± SD values.

Comparative Hydrolysis Studies

Hydrolysis studies by NMR included monitoring hydrolysis of the 2,6-dimtheylphenoxo groups where applicable as previously described.[5–7] The complexes were separately dissolved in d_6-DMSO solutions and D_2O was added to give final solution of 1:9 D_2O/d_6-DMSO with added D_2O being ca.1000 equiv relative to the Ti(IV) complex. The $t_{1/2}$ values were determined based on pseudo first-order fit for each compound, as the average of values obtained for several signals of the labile ligands. The results were verified by including *p*-dinitrobenzene as an internal standard.

Live Cell Confocal Imaging

HeLa cells (1.68×10^5 cells) were seeded in µ-slide 8 well (ibidi) with 300 µL per well of culture medium and allowed to attach for a day under incubation at 37 °C in 5% CO_2 atmosphere. After treatment with Hoechst® (0.1 µM) and green or deep red Mitotracker® (1 µM) for 0.5 hour incubation, the staining solution was washed out and replaced with fresh culture medium. The cells were then treated with the Ti(IV) compounds (5 µM for the mononuclear compounds and 2 µM for the hydrolysis products in max. 0.5% DMSO). Cells were imaged using the FV-1200 confocal microscope (Olympus, Japan). Multiple dyes sequential scanning mode was used to avoid emission bleed-through.

Synthesis and Characterization

1b; 1a[1] (172 mg, 0.376 mmol) in dry CH_2Cl_2 was stirred until dissolution was achieved. $Ti(OArMe_2)_4$[2] (200 mg, 0.376 mmol) dissolved in dry CH_2Cl_2 was added and the solution was left to stir 3 days at room temperature under a nitrogen atmosphere. The crude product was evaporated under vacuum, washed several times with dry hexane, and evaporated again under vacuum to give a dark green-brown product (209 mg, 74 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.23 (s, 2 H, CH), 7.51 (d, J = 9.4 Hz, 2 H, Ar), 6.69 (d, J = 7.4 Hz, 2 H, Ar), 6.39 (t, J = 7.4 Hz, 2 H, Ar), 5.70 (t, J = 2.1 Hz, 2 H, Ar), 3.44 (q, J = 7.0 Hz 8 H, CH2), 2.05 (s, 12 H, CH3), 1.10 (t, J = 7.0 Hz, 12 H, CH3) ppm.^{13}C NMR (125 MHz, $CDCl_3$): δ = 186.8, 164.6, 156.9, 155.1, 152.3, 138.0, 128.8, 127.8, 126.4, 118.8, 113.4, 111.7, 96.6, 45.4, 17.7, 13.0 ppm. Anal. Calc. for $C_{42}H_{46}N_6O_4Ti$: C, 67.56, H, 6.21, N 11.25. Found: C, 66.90, H, 6.02, N, 11.65.

2b; 2b was synthesized similarly from **2a**[1] (172 mg, 0.376 mmol) and $Ti(OArMe_2)_4$[2] (200 mg, 0.376 mmol) in THF to give red product (239 mg, 85%). ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.75 (s, 2 H, CH), 7.71 (m, 2 H, Ar), 7.40 (d, J = 9.1 Hz, 2 H, Ar), 7.26 (m, 2 H, Ar), 6.56 (d, J = 7.4 Hz, 4 H, Ar), 6.26 (t, J = 7.4 Hz, 2 H, Ar), 6.23 (dd, J = 9.0 Hz, 2.3, 2 H, Ar), 5.72 (d, J = 2.2 Hz, 2 H, Ar), 3.37 (q, J = 7.0 Hz, 8 H, CH2), 1.90 (s, 12 H, CH3), 1.10 (t, J = 7.0 Hz, 12 H, CH3) ppm.^{13}C NMR (125 MHz, $CDCl_3$): δ = 167.8, 164.5, 155.2, 155.0, 143.6, 136.8, 128.7, 127.3, 127.0, 126.5, 117.6, 115.7, 114.0, 104.8, 97.1, 45.0, 17.5, 12.9 ppm. Anal. Calc. for $C_{44}H_{50}N_4O_4Ti$: C, 70.77, H, 6.75, N 7.50. Found: C, 70.32, H, 6.61 , N, 7.38.

Crysal data for 2b; $C_{45}H_{52}Cl_2N_4O_4Ti$, M = 831.71, Monoclinic, a = 16.232(3), b = 16.059(3), c = 16.430(3)Å, $\alpha = \gamma = 90$, β = 96.520(3)°, V = 4255.1(1) Å^3, T = 123(1) K, space group C2/c, Z = 4, μ(Mo-Kα) = 0.374 mm^{-1}, 22391 Reflections measured, 4635 unique (R_{int} = 0.0413), $R(F_o^2)$ for [I>2σ(I)] = 0.0607, Rw for [I>2σ(I)] = 0.1306.

1c; 1b in THF was stirred with 10,000 equiv of water in THF at RT for 3 days. The crude product was evaporated under vacuum to give a red product quantitatively. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.29 (s, 4 H, CH), 7.56 (d, J = 9.0 Hz, 4 H, Ar), 6.45 (d, J = 9.0 Hz, 4 H, Ar), 6.02 (s, 4 H, Ar), 3.44 (q, J = 7.0 Hz, 8 H, CH2), 1.18 (t, J = 7.0 Hz, 24 H, CH3) ppm.

2c; 2b in THF was stirred with 10,000 equiv of water in THF at RT for 3 days. The crude product was evaporated under vacuum to give a red product quantitatively. ^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.87 (s, 4 H, CH), 7.85 (m, 4 H, Ar), 7.51 (d, J = 9.1 Hz, 4 H, Ar), 7.31 (d, 4 H, Ar), 6.34 (dd, J = 8.8 Hz, 2.4 Hz, 4 H, Ar), 6.05 (d, J = 2.4 Hz, 4 H, Ar), 3.46 (q, J = 6.7 Hz, 16 H, CH2), 1.17 (t, J = 6.7 Hz, 24 H, CH3) ppm. ESI (m/z): $[M^+H]^+$ calcd. for $C_{56}H_{64}N_8O_6Ti_2$, 1041.3994; found 1041.40251.

Supporting Figures and Tables

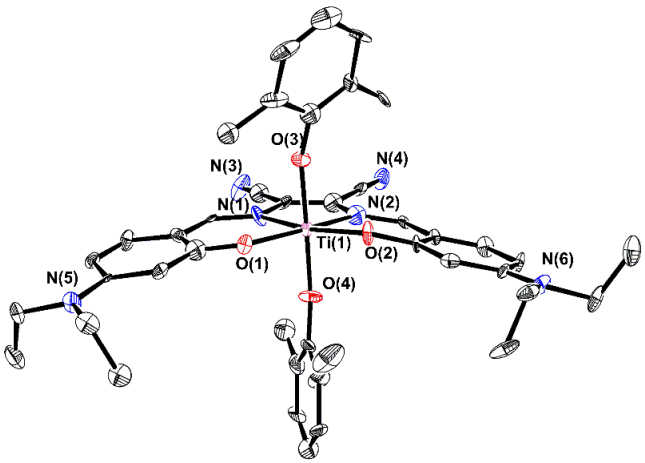


Figure S1. ORTEP drawing of **1b**, with 50% probability ellipsoids; H atoms were omitted for clarity.

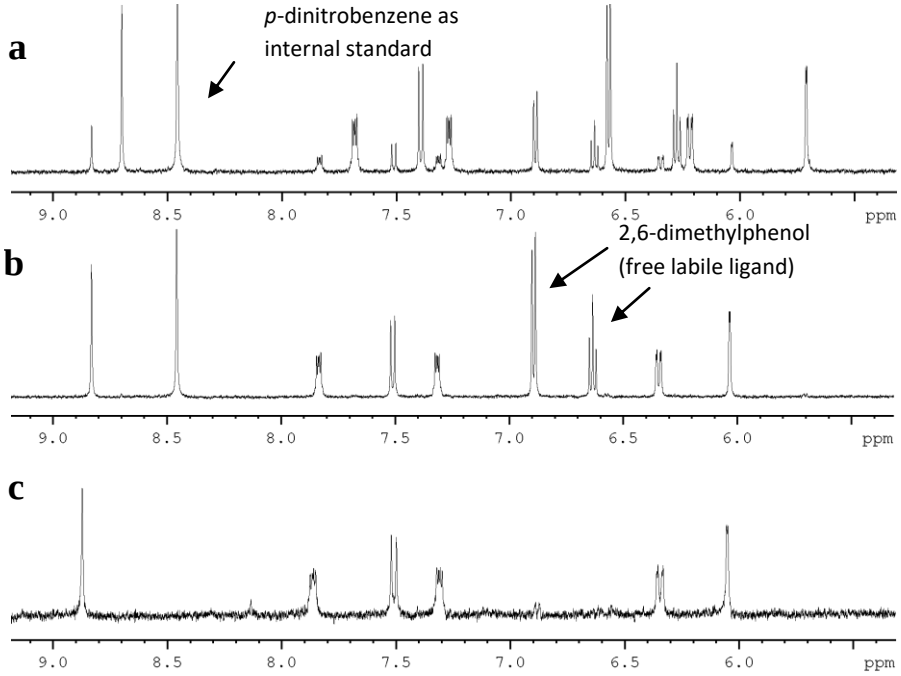


Figure S2. ^{1}H-NMR spectra of the aromatic region of **2b**, undergoing hydrolysis at room temperature in d_6-DMSO (a) three minutes and (b) 54 minutes after the addition of 10% (1000 equiv) D_2O, with an internal standard; and (c) 3 days after the addition of 10000 equiv of H_2O.

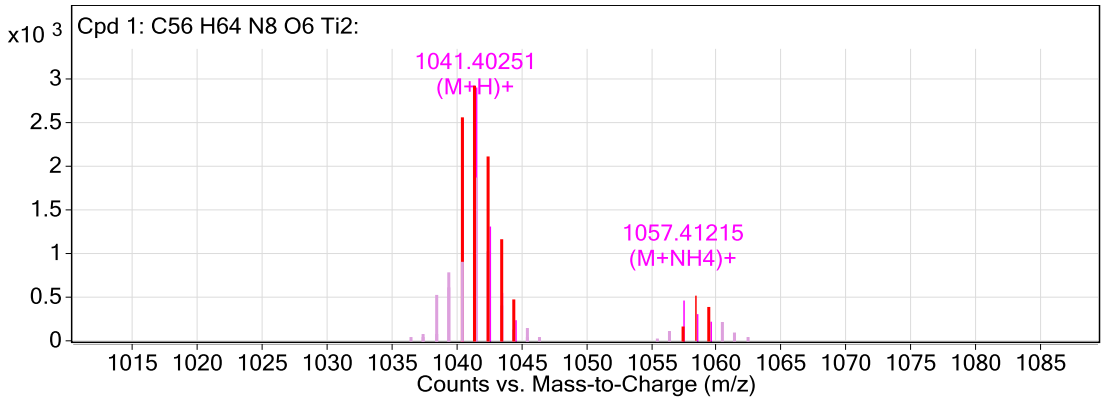


Figure S3. ESI mass spectra recorded for **2c**, calculated m/z 1041.39939, recorded m/z 1041.40251.

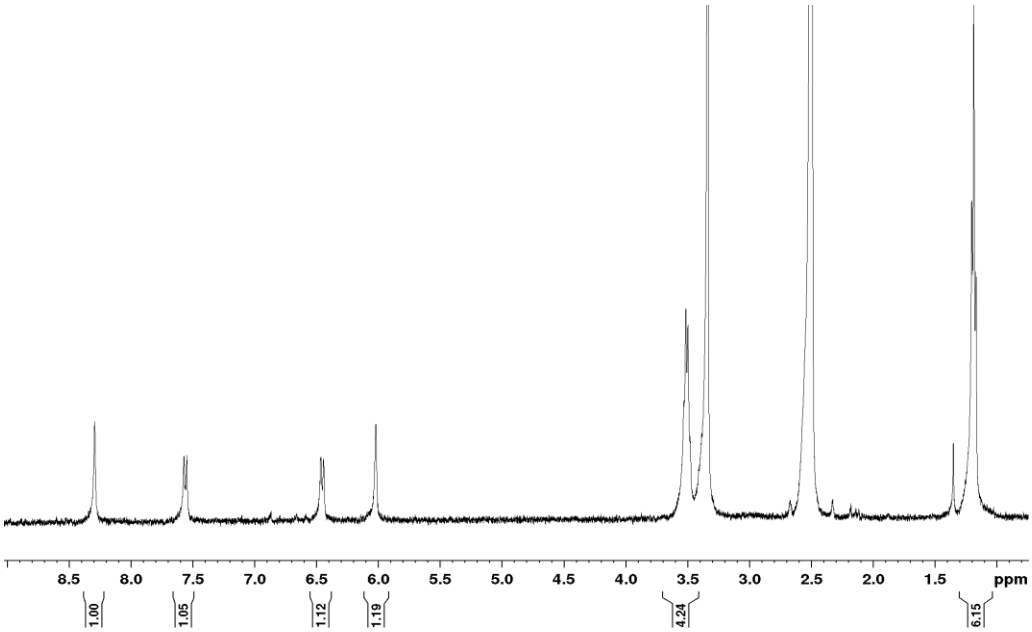


Figure S4. ^{1}H-NMR spectra of **1c** at room temperature in d_6-DMSO three days following addition of > 10000 eq. of H_2O.

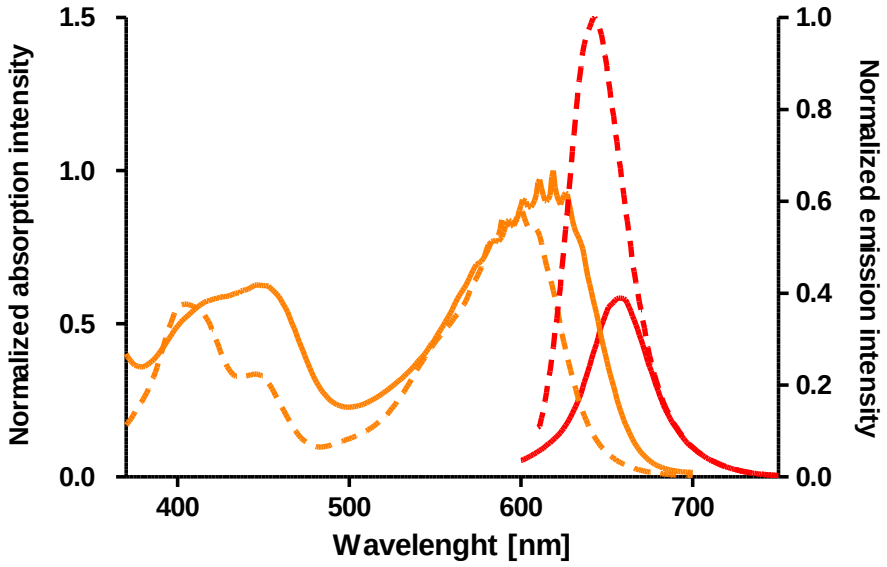


Figure S5. Normalized absorption (orange) and emission (red) spectra in DMSO at room temperature of **1b** (excited at 580 nm, full lines) and its hydrolysis product **1c** (excited at 550 nm, dash lines) both at a concentration of 20 µM.

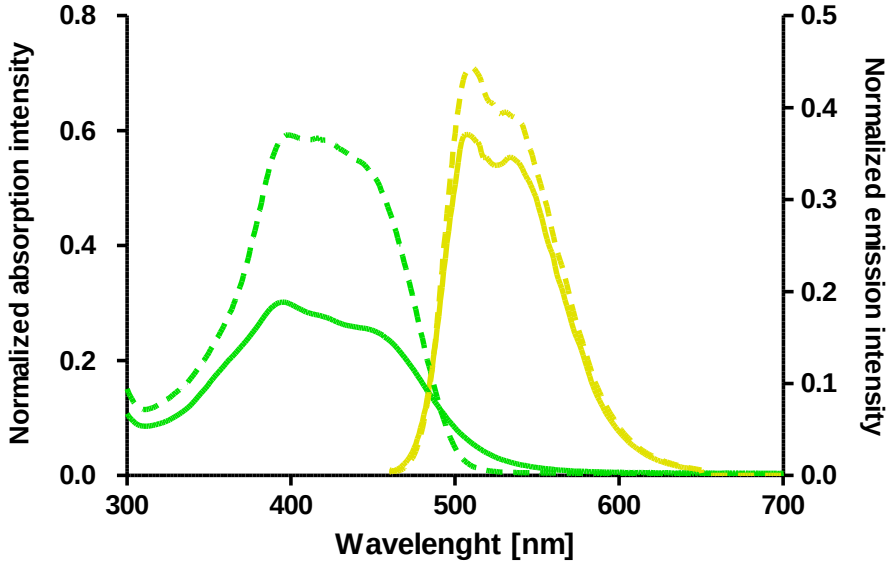


Figure S6. Normalized absorption (green) and emission (yellow) spectra in DMSO at room temperature of **2b** (excited at 405 nm, full lines) and its hydrolysis product **2c** (excited at 450 nm, dash lines) both at a concentration of 20 µM.

Table S1. Fluorescence quantum yield of the Ti(IV) compounds[a]

Compound Name:	Φ[b]
1b	0.111 excited at 590 nm
2b	0.053 excited at 455 nm
1c	0.598 excited at 550 nm
2c	0.126 excited at 455 nm

[a] all data determined in DMSO at a concentration of 20 μM.

[b] Fluorescence quantum yield

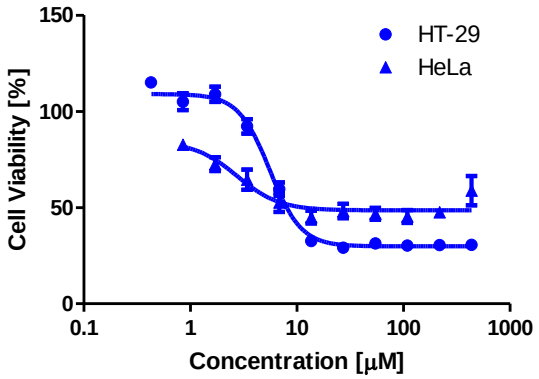


Figure S7. Dependence of HT-29 and HeLa cell viability (based on MTT assay after a 3 days incubation period) on added concentration of the free ligand **2a**, presented on a logarithmic scale.

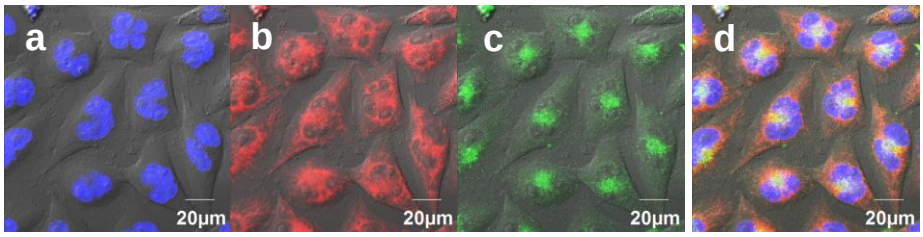


Figure S8. Merged fluorescence colocalization images with DIC image of HeLa cells incubated with **2a** for 1 hour (5 μM with 0.2% DMSO in medium) and commercial dyes;

(a) image of Hoechst®, (b) image Mytotracker®, (c) image of **2a**, (d) merged images of (a)-(c).

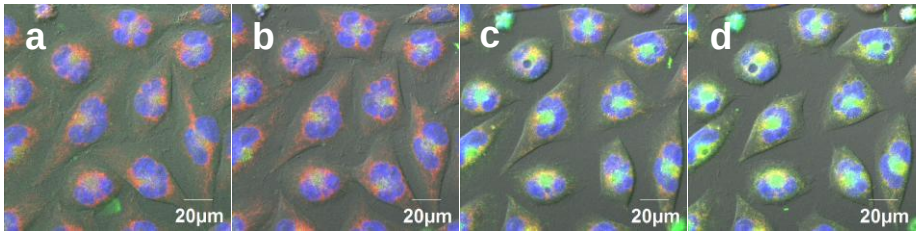
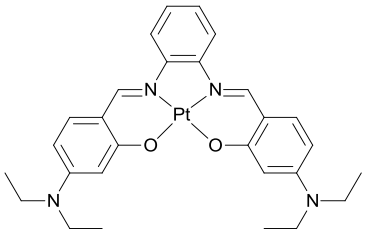


Figure S9. Merged fluorescence colocalization images with DIC image of HeLa cells incubated with **2a** 0.5 (a), 1 (b), 3 (c), and 5 (d) hours with continuous radiation (5 μM with 0.2% DMSO in medium) all with commercial dyes; blue for Hoechst®, red for Mitotracker® and green for **2a**.



Scheme S1. 2a-Pt complex that was investigated as control.

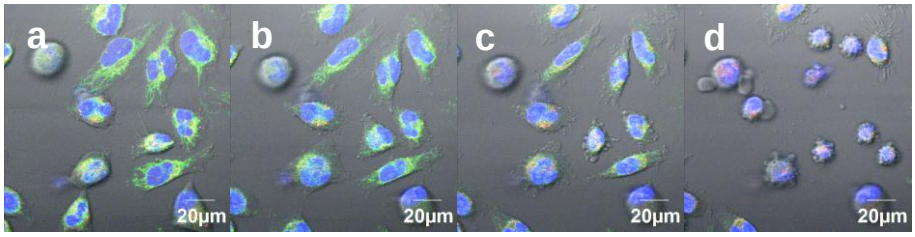


Figure S10. Merged fluorescence colocalization images with DIC image of HeLa cells incubated with **2a-Pt** for 5 minutes (a), and 1 (b), 2 (c), and 3 (d) hours with continuous radiation, (2 μM with 0.5% DMSO in medium) all with commercial dyes; blue for Hoechst®, green for Mitotracker® and red for **2a-Pt**.

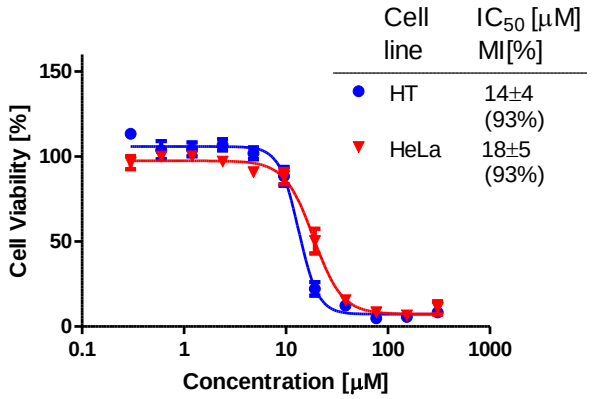


Figure S11. Dependence of HT-29 and HeLa cell viability (based on MTT assay after a 3 days incubation period) on added concentration of **2a-Pt** presented on a logarithmic scale.

References:

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