Fluorescent Gallium and Indium *bis*(thiosemicarbazonates) and their Radiolabelled Analogues: Synthesis and Cellular Confocal Fluorescence Imaging Investigations

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S1-General Experimental

All reagents were purchased from Sigma-Aldrich, Merck Chemicals, Fluorous Technologies or Alfa-Aesar and were used as supplied without prior purification unless otherwise stated. ^{1}H NMR spectra were recorded on a Varian Mercury VX300 (300 MHz) spectrometer or a Varian Unity (500 MHz) spectrometer or a Bruker AVC 500 (500 Hz) spectrometer at 298 K and referenced to residual non-deuterated solvent peaks. Chemical shifts are quoted in ppm with signal splittings recorded as singlet (s), doublet (d), triplet (t), quartet (q), quintet (qt) and multiplet (m). Coupling constants, *J*, are measured to the nearest 0.1 Hz and are presented as observed. ^{13}C NMR spectra were recorded on a Varian Mercury VX300 (300 MHz) spectrometer or a Varian Unity (500 MHz) spectrometer or a Bruker AVC 500 (500 Hz) spectrometer at 298 K and were referenced to the solvent peak.
Mass spectrometry was performed using a Bruker Micromass LCT instrument on a Micromass LCT time-of-flight mass spectrometer under conditions of electrospray ionization (ESI-MS). Accurate masses are reported to four decimal places using tetraoctylammonium bromide (466.5352 Da) as an internal reference. Values are reported as a ratio of mass to charge in Daltons. HPLC characterisation (radio-HPLC) of compounds was performed by the following method:
Radio HPLC was performed using a Waters C-18 column (4.6 x 250 mm) with UV/vis detection at λ_{obs} = 254 nm with a 1.0 mL/min gradient elution method (Solvent A: acetonitrile with 0.1 % TFA *v/v*, Solvent B: water with 0.1% TFA *v/v*): start 5 % A, gradient over 10 min reaching 95 % A, hold to 12 min at 95 % A, reverse gradient till 14 min reaching 5 % A, then hold to 15 min at 5 % A.

Elemental analyses were performed either by the microanalysis service at the Chemistry Department, University of Oxford, or by Mr. S. Boyer, at London Metropolitan University.

Electronic absorption spectroscopy (UV/vis) was performed using a Perkin-Elmer Lambda 19 spectrometer, running UV Winlab software. Spectra were measured using 1.00 cm quartz cuvettes. Transmetallation reactions were performed by the addition of 10 x 0.1 equivalent of $GaCl_3$ in Methanol to a solution of the desired zinc complex.

Fluorescence spectra were recorded in 1.00 cm quartz cuvettes using a Hitachi F-4500 fluorescence spectrometer, running FL Solutions software. Relative quantum yields were determined by comparison to either Fluorescein in 0.1 M NaOH (Φ_R = 0.95 at 496nm) or $[Ru(bipy)_3](PF_6)_2$ in water (Φ_R = 0.042 in water at 420 nm), using the following formula:

$$\Phi_S = \Phi_R \cdot (D_S/D_R) \cdot (A_R/A_S) \cdot (I_R/I_S) \cdot (\eta_S/\eta_R)^2$$

Φ is the relative quatum yield, D is the integrated area of the fluorescence emission peak, A is the absorption of the solutions at the excitation wavelength, I is the flux at the excitation wavelength used and η is the solution refractive index. R and S subscripts refer to the reference and sample respectively

Microscopy studies

Cells were seeded as monolayers in T75 tissue culture flasks, and HeLa were cultured in Eagle's Minimum Essential Medium (EMEM) and PC3 in RMPI-1640 each supplemented with 10 % foetal bovine serum, L-glutamine, penicillin and streptomycin. Cells were maintained at 37 °C in a 5 % CO_2 humidified atmosphere and grown to approximately 85 % confluence before being split using 2.5 % trypsin. For microscopy, cells were seeded onto chambered coverglass slides and incubated for 12 h to ensure adhesion.

Bis(thiosemicarbazone) complexes were prepared as 10 mM solution in DMSO and diluted to 100 or 50 μM with EMEM and incubated with the cells at 37 °C or 4 ^{o}C. Prior to imaging, the solution was replaced with 1 mL fresh EMEM. Background autofluorescence was measured by imaging the cells in 1 mL of EMEM medium only.

The fluorescent uptake of the *bis*(thiosemicarbazone) complexes was imaged by laser-scanning confocal microscopy (TCS NT, Leica), using the 488 nm line of an argon ion laser for excitation and the emission was long pass filtered (515 nm).

Fixed cell imaging

Cells were plated in a Petri dish containing a glass cover slip. Before fluorescence imaging, the serum containing medium was replaced with SFM. After addition of compound, cells were fixed by inverting the coverslip placing it onto 50 μl of paraformaldehyde-PBS (4% paraformaldehyde). After 15 min, the coverslip was mounted to a slide using Vectashield®, hard set mounting medium with DAPI (Vector Laboratories, Inc.)

S2-X-ray Crystallography

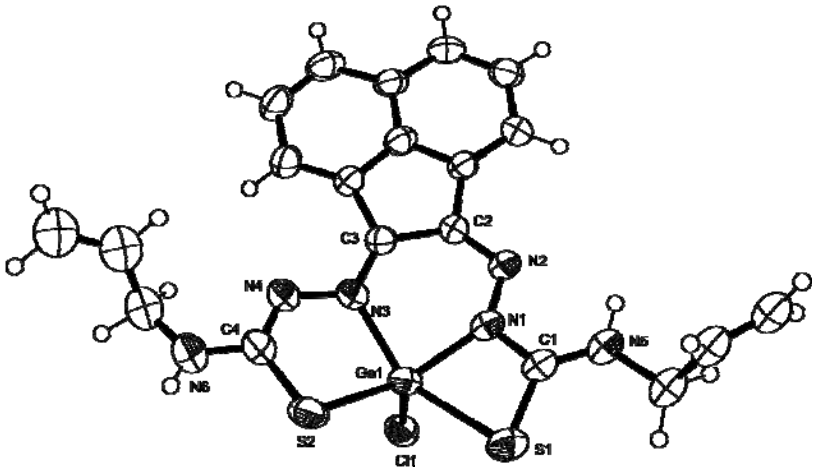

Figure S2.1. ORTEP representation (50% ellipsoid probability) of **4b**.

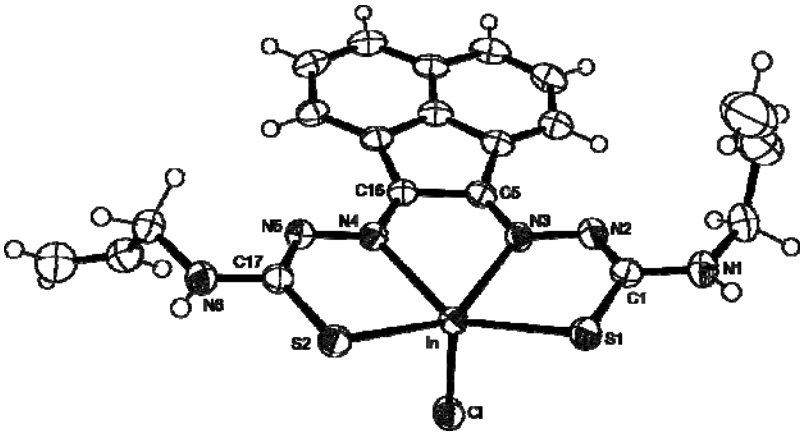

Figure S2.21. ORTEP representation (50% ellipsoid probability) of **6**.

S3-General ^{111}In Radiolabelling Procedure

The ligand or complex was prepared as a 1.0 mg/ml solution in DMSO or distilled water. For indium labeling 10 µl of the stock solution was diluted with 90 µl of pH 4.5 sodium acetate buffered solution and heated at 60 °C for 15 min in an Eppendorf tube in the presence of 20 µl $^{111}InCl_3$ (< 10 MBq per experiment).

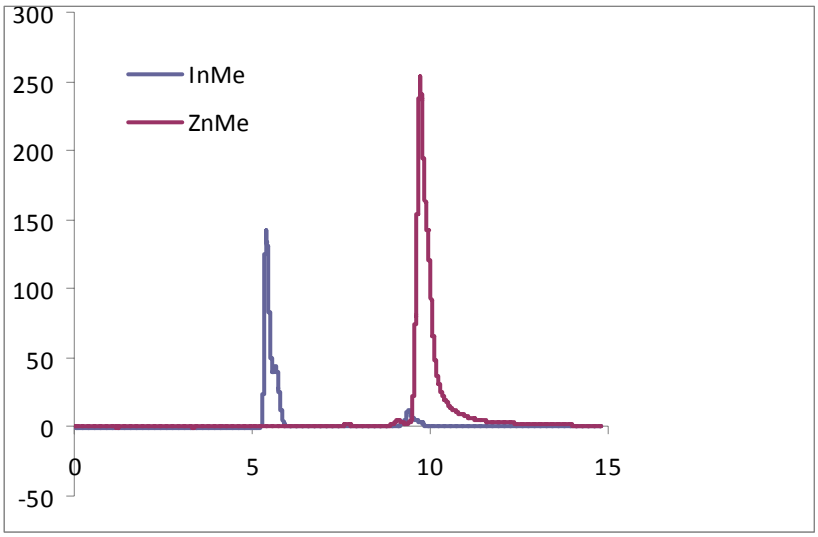

Figure S3.1: Overlay of UV-HPLC trace (maroon) of aromatic zinc *bis*(thiosemicarbazone) precursor **C** (R=methyl) with the radio-HPLC trace (purple) of aromatic ^{111}InCl *bis*(thiosemicarbazonato) complex **5**, [^{111}In]**5**.

Measurement of LogP

The indium-111 complex **5** or **6** (15 µL, <1MBq) was added to a mixture of octan-1-ol (0.5 mL) and H_2O (0.5 mL). The mixture was shaken for 1 min, then centrifuged for 5 min at 2000 rpm. A 50 µL sample of each layer was taken and counted using a gamma counter. The measurements were performed in triplicate. Log *P* was calculated using the formula:

$\log P = \log(\underline{\text{counts(octanol)}}/\text{counts}(H_2O))$

S4-Ga-68 radiolabelling experiments:

Materials and methods

Analytical HPLC was performed with a system from Bischoff Analysentechnik (Leonberg, Germany) equipped with a Lambda 1010 UV-detector and a Berthold LB-506 radiodetector using a Machery-Nagel Nucleodur Isis C_{18}-column (5µ, 250×4.6 mm, eluents: A, 0.1% trifluoroacetic acid (TFA) in water and B, acetonitrile (MeCN); gradient, 0-7.5 min, 95-5% A; 7.5-12.5 min, 5% A; 12.5-15 min 5-95% A; 15-17.5 min, 95% A; flow: 2 mL/min).

HPLC-chromatogramms of reference compound and labelling precursor:

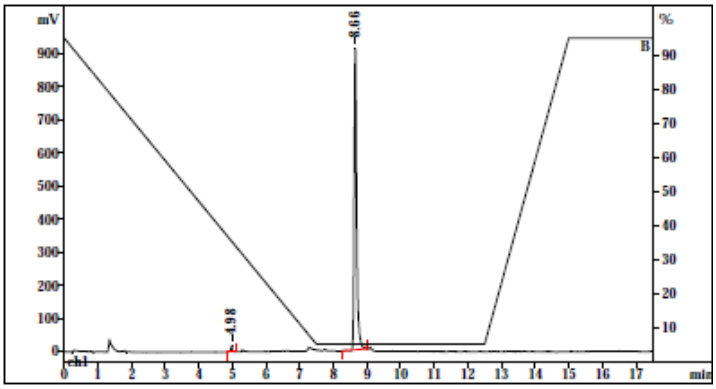

Figure S4.1: UV-HPLC of Precursor **D** "Zn-Allyl"

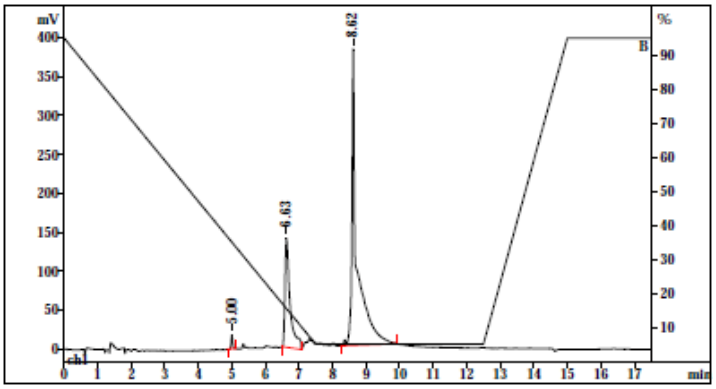

Figure S4.2: UV-HPLC of **4** "Ga-Allyl"

The presence of both isomers – symmetric and asymmetric is observable in solution, consistent with the ^{1}H-NMR data (Figure S4.2)

Elution and purification of ^{68}Ga

^{68}Ga was obtained from a 30 mCi ^{68}Ge/^{68}Ga-Generator (Cyclotron Co. Ltd, Obninsk, Russia) connected to an Eckert&Ziegler ModularLab system which was used for elution, purification and concentration of the ^{68}Ga-eluate. The method applied is based on the cationic pre-purification strategy developed by Zhernosekov[1] and Ocak.[2] Briefly, the generator was eluted with 7 mL of 0.1 M HCl (Ultrapure-Grade) and the eluate was loaded on a StrataX-C cationic exchange column (Phenomenex, 33 mg, 1 mL). Pre-purified

$^{68}GaCl_3$ (435 MBq, 11.75 mCi) was eluted with 700 µL of 98% acetone/0.02 M HCl solution.

*Radiolabelling of Zn-Allyl '**D**'*

All labelling reactions were carried out with ~1 mCi (~37 MBq) $^{68}GaCl_3$. We applied two different labelling procedures using either acetate/Tris-buffer (110 mM/11 mM) or HEPES-solution (0.5 M, pH 3.9). Following procedure 1, to 60 µL ^{68}Ga in 98% acetone/0.02 M HCl was added 640 µL 98% acetone/0.02 M HCl, 540 µL de-ionized H_2O, 140 µL acetate/Tris-buffer and 50 µL Zn-Allyl '**D**' (1 mg/mL in DMSO). According to procedure 2, to 60 µL ^{68}Ga in 98% acetone/0.02 M HCl was added 640 µL 98% acetone/0.02M HCl, 1 mL 0.5 M HEPES solution and 50µL Zn-Allyl '**D**' (1 mg/mL in DMSO). With both procedures degradation of Zn-Allyl '**D**' occurred which was confirmed by inactive reactions under the same conditions (Figures S4.3-S4.6).

For a successful labelling reaction (procedure 3), 60 µL ^{68}Ga in 98% acetone/0.02 M HCl was added to a glass vial and evaporated to dryness with a stream of nitrogen in a heating block at 90°C for 20 min. Subsequently 500 µL EtOH and 50 µL Zn-Allyl '**D**' (1 mg/mL in DMSO) were added to the vial and heated for 30 min at 90 °C.

For quality control 25 µL labelling solution were diluted with 50 µL of 0.1 mM DTPA in 0.1 M sodium acetate buffer (pH 5.2) and 20 µL were injected into HPLC. The retention time was 1.3 min for ^{68}Ga-DTPA and 9.1 min for ^{68}Ga-Allyl '[^{68}Ga]**4**'. The radiochemical purity was 71.2 %. (Figure S4.7 and S4.8)

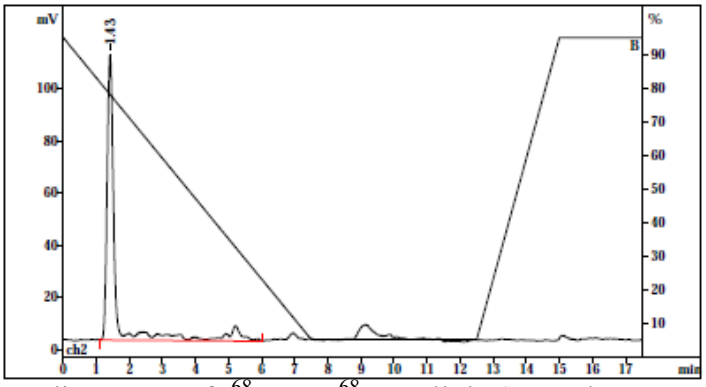

Figure S4.3: radio-HPLC of [^{68}Ga]**4** "^{68}Ga-Allyl" (procedure 1, 90°C, 30 min)

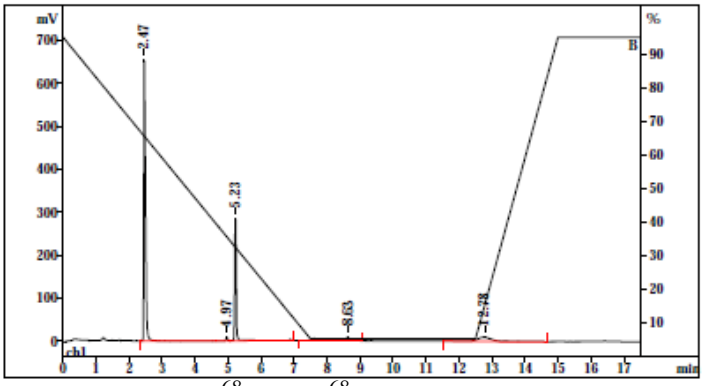

Figure S4.4: UV-HPLC of [^{68}Ga]**4** "^{68}Ga-Allyl" (procedure 1, 90°C, 30 min)

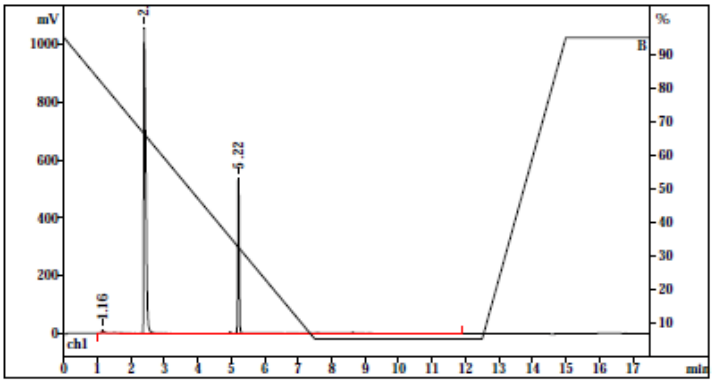

Figure S4.5: UV-HPLC of degradation of precursor **D** "Zn-Allyl"

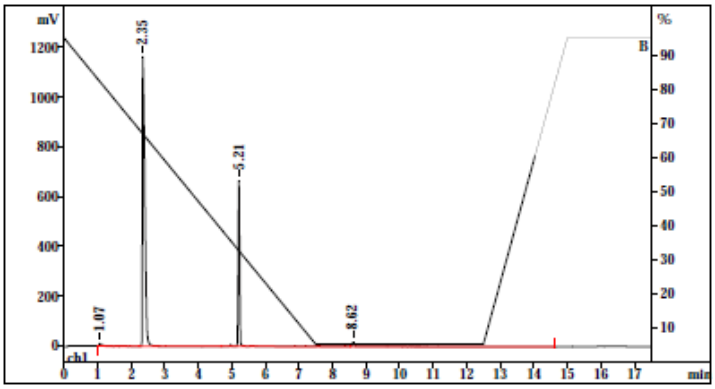

Figure S4.6: UV-HPLC of degradation of precursor **4** "Ga-Allyl"

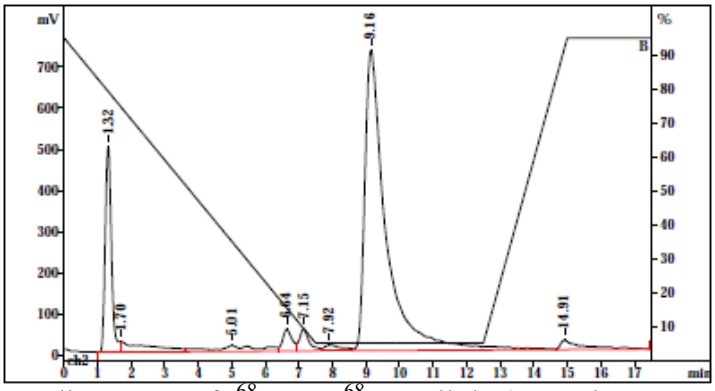

Figure S4.7: radio-HPLC of [^{68}Ga]**4** "^{68}Ga-Allyl" (procedure 3, 90°C, 30 min)

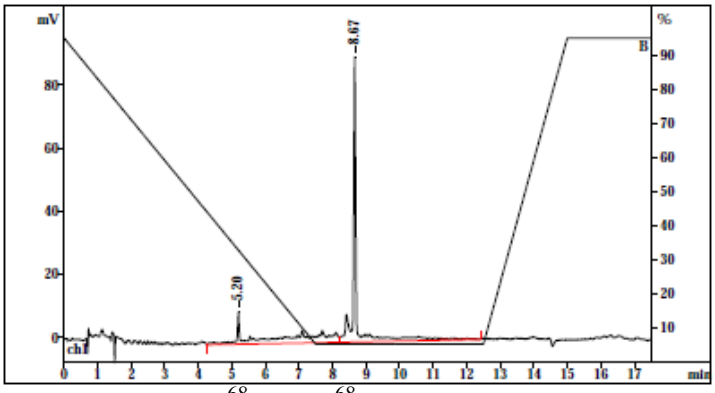

Figure S4.8: UV-HPLC of [^{68}Ga]**4** "^{68}Ga-Allyl" (procedure 3, 90°C, 30 min)

To verify whether the peak at ca. 6 min retention time is an isomeric form of the product, we collected the main peak at ca. 9 min retention time and re-injected it into the HPLC (Figures S4.9 and S4.10). Indeed the peak at 6 min was formed again, and this is consistent with "cold" experiments and spectroscopic investigations which suggested that this compound is likely to be present as a mixture of sym/asym isomers in solution.

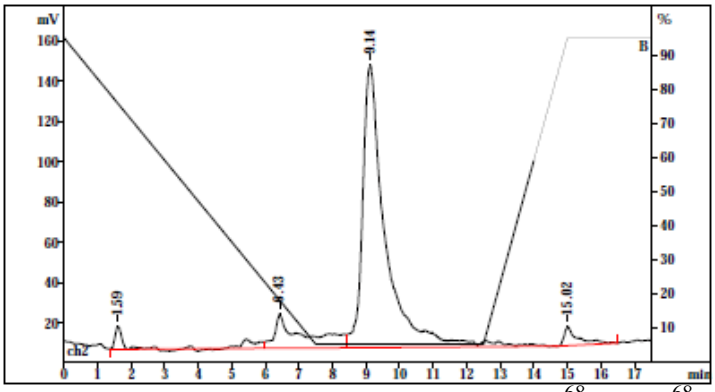

Figure S4.9: radio-HPLC of reinjection of isolated [^{68}Ga]**4** "^{68}Ga-Allyl"

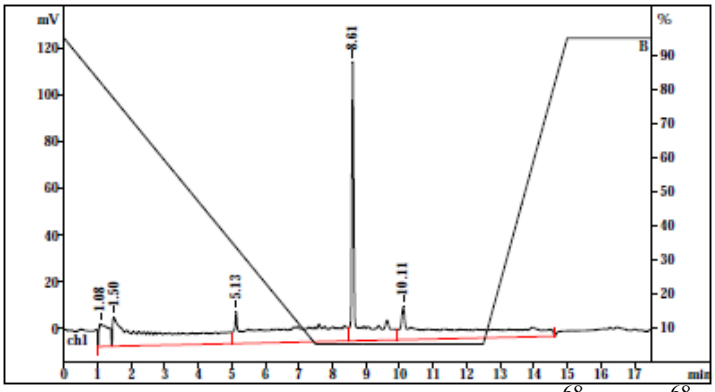

Figure S4.10: UV-HPLC of reinjection of isolated [^{68}Ga]**4** "^{68}Ga-Allyl"

1. K. P. Zhernosekov, D. V. Filosofov, R. P. Baum, P. Aschoff, H. Bihl, A. A. Razbash, M. Jahn, M. Jennewein, F. Roesch, *J. Nucl. Med.*, 2007, **48**, 1741-1748
2. M. Ocak, M. Antretter, R. Knopp, F. Kunkel, M. Petrik, N. Bergisadi, C. Decristoforo, *Appl. Radiat. Isotopes*, 2010, **68**, 297.

S5-*in vitro* fluorescence imaging

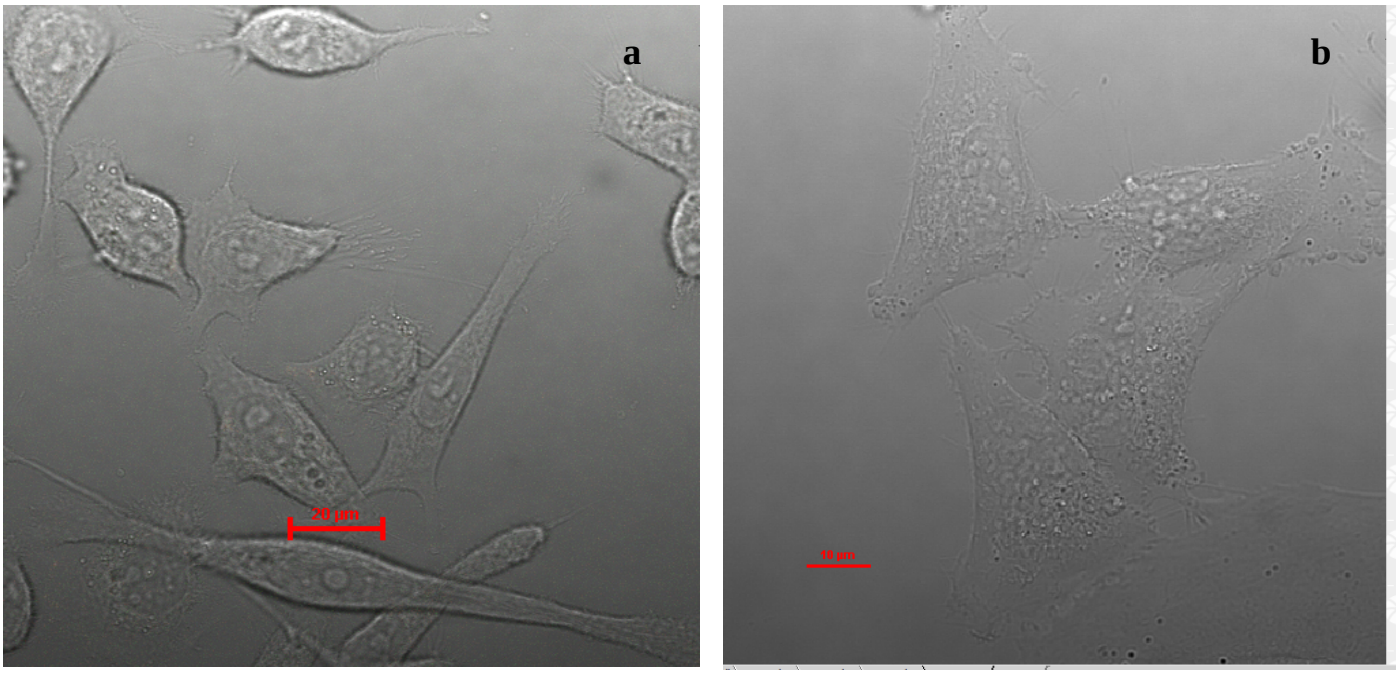

Figure S5.1 Control experiments – PC3 (**a**) and HeLa (**b**) cells prior to imaging, after 20 min incubation 37 ^{o}C in SFM with 1% DMSO and no compound added (DIC images)

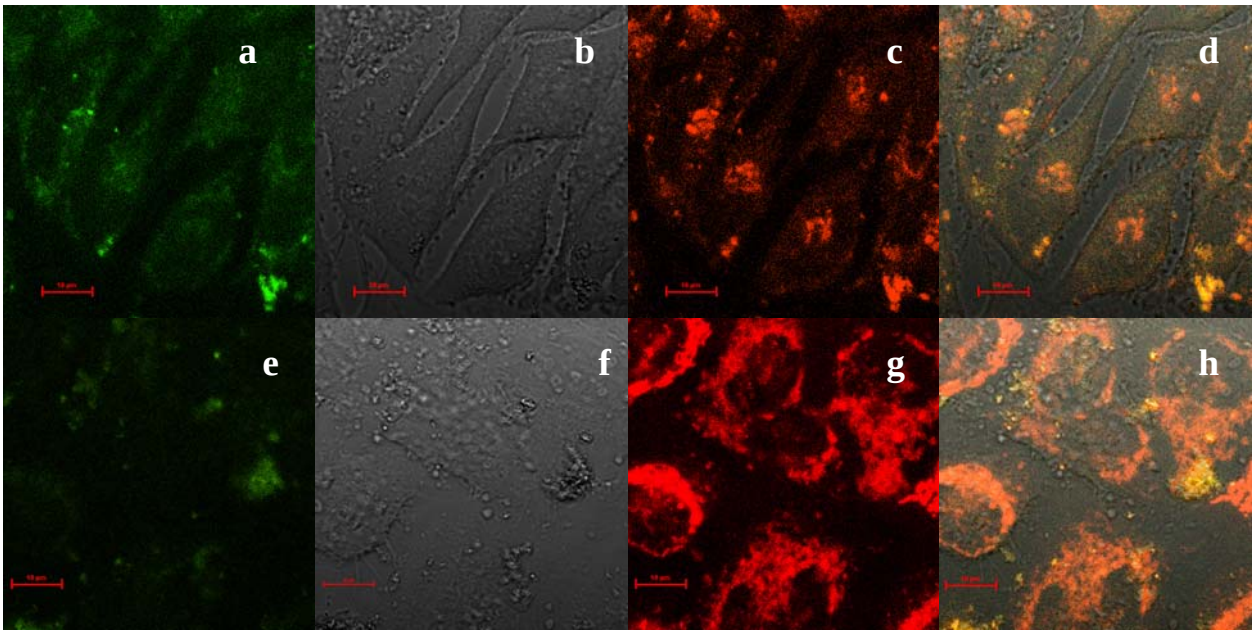

Figure S5.2 Imaging of free pro-ligand **L3** and co-localisation with dyes.
(a)-(d): **L3** uptake in HeLa cells, co-localisation with Lysotracker Red: a) ex 488 nm, em. 516-530 nm
b) DIC image c) ex 543 nm, emission 605-675nm, d) overlay of (a)-(c) micrographs
(e)-(h): **L3** uptake in HeLa cells, co-localisation with Mitotracker Red: e) ex 488 nm, em. 516-530 nm
f) DIC image, g) ex 543 nm, emission 605-675nm, h) overlay of (a)-(c) micrographs
Note: No fluorescence was observed at 50 µM concentration 1% DMSO, after 20 min or 1 h incubation at 37 ^{o}C.
These images were recorded using 100 µM conc in 1% DMSO, after 20 min incubation 37 ^{o}C in SFM with 1% DMSO.

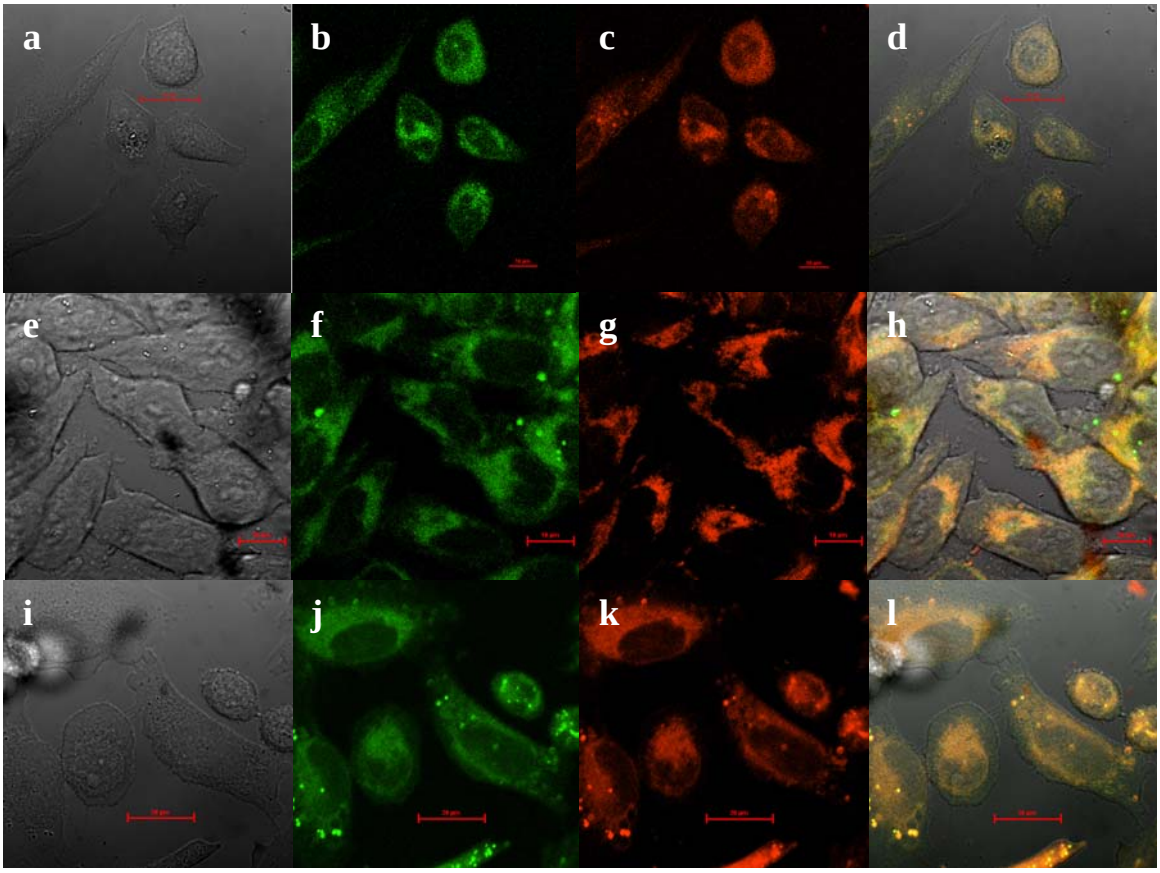

Figure S5.3 Brightfield and Laser scanning confocal fluorescence microscopy images at 50 μM conc, 1% DMSO
(a-d) Complex **3** in PC3, incubation time 20 min, 37 ^{o}C: (a) DIC image, b) ex 488 nm, emission 516-530 nm, c) Lysotracker colocalisation: ex 543 nm, em 605-675 nm, d) overlay (a-c); (e-h) Complex **3** in HeLa, incubation time 1 h, 37 ^{o}C; (e) DIC image, f) ex 488 nm, emission 516-530 nm, g) Mitotracker colocalisation: ex 543 nm, em 605-675 nm, h) overlay (e-g).; (i-l) Complex **4** in HeLa, incubation time 1 h, 37 ^{o}C; (i) DIC image, j) ex 488 nm, emission 516-530 nm, k) Lysotracker colocalisation: ex 543 nm, em 605-675 nm, l) overlay (e-g).

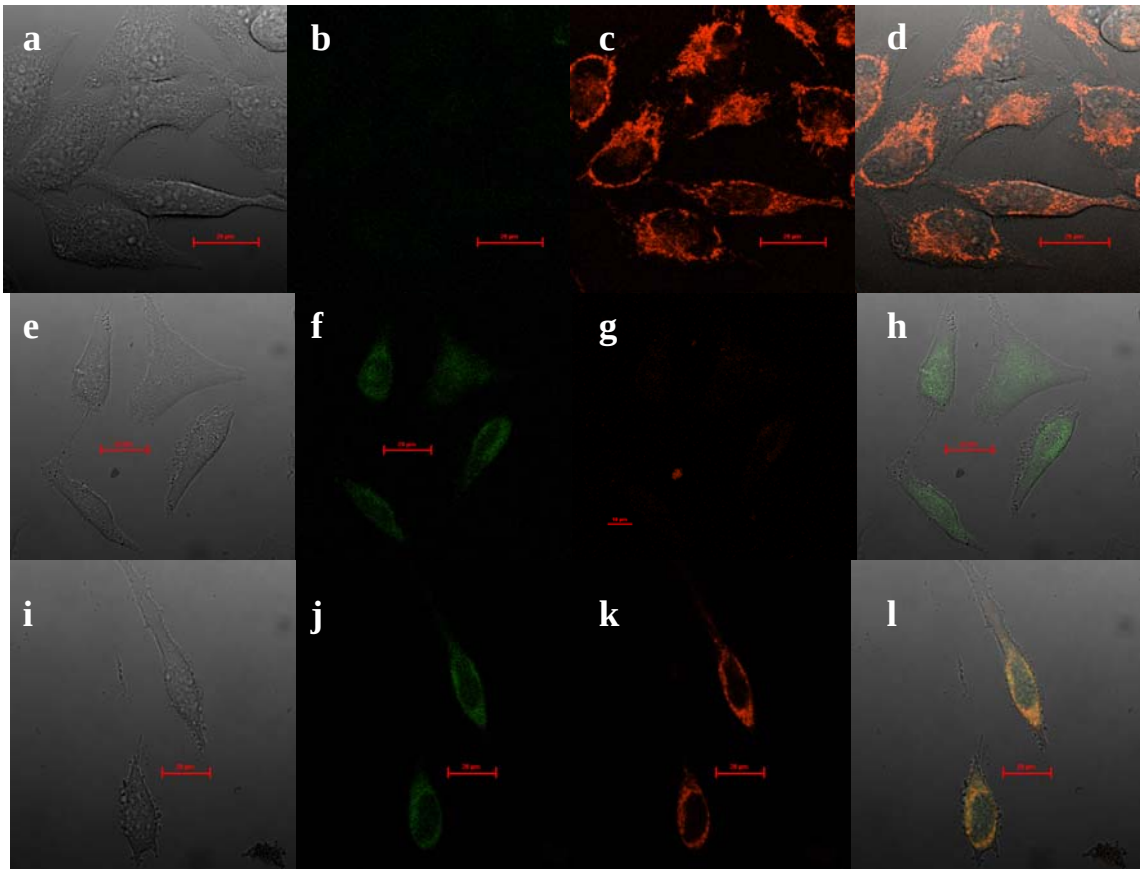

Figure S5.4 Brightfield and Laser scanning confocal fluorescence microscopy images t 50 μM conc, 1% DMSO:
(a-d) Control experiments in HeLa incubation time 20 min, 37 ºC: (a) DIC image, b) ex 488 nm, emission 516-530 nm, c) Mitotracker stain: ex 543 nm, em 605-675 nm, d) overlay (a-c); (e-h) Complex **3** in HeLa, incubation time 20 min, 37 ºC; (e) DIC image, f) ex 488 nm, emission 516-530 nm, g) ex 543 nm, em 605-675 nm, h) overlay (e-g).; (i-l) Complex **3** in HeLa, incubation time 20 min, 37 ºC; (i) DIC image, j) ex 488 nm, emission 516-530 nm, k) Mitotracker colocalisation: ex 543 nm, em 605-675 nm, l)

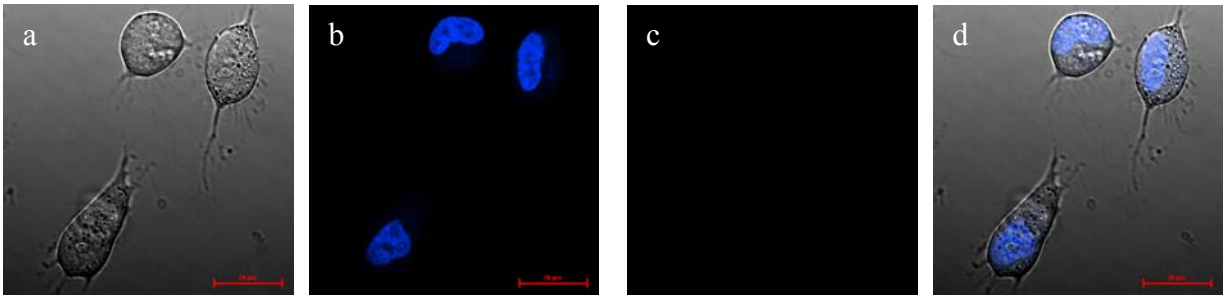

Figure S5.5: 1-photon confocal microscopy images of Hoechst in HeLa, (a) DIC image, (b) excitation at 405 nm emission at 451 (c) excitation at 488 nm, emission 516 nm, (c) an overlay of the previous three images

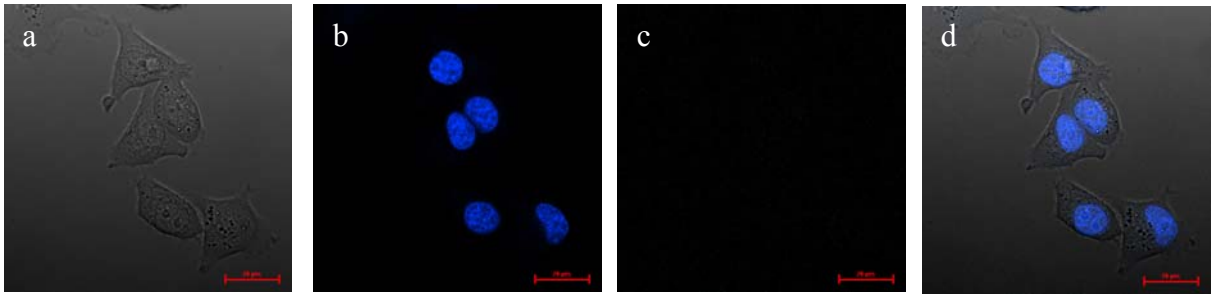

Figure S5.6: 1-photon confocal microscopy images of Hoechst in PC3, (a) DIC image, (b) excitation at 405 nm emission at 451 (c) excitation at 488 nm, emission 516 nm, (c) an overlay of the previous three images

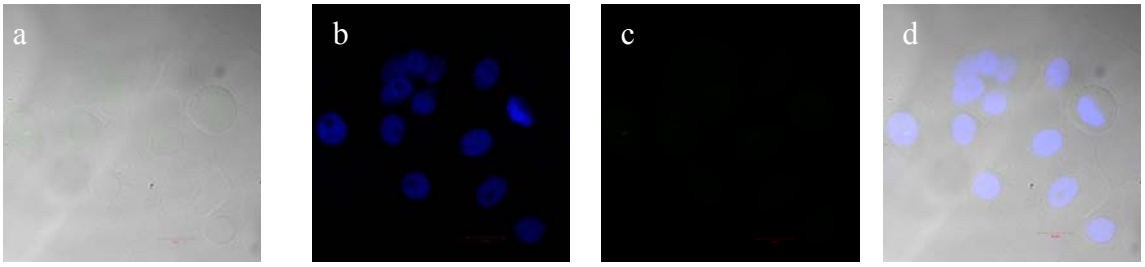

Figure S5.7: 1-photon confocal microscopy images of DAPI in HeLa (fixed cells), (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) overlay of (a)+(b)+(c)

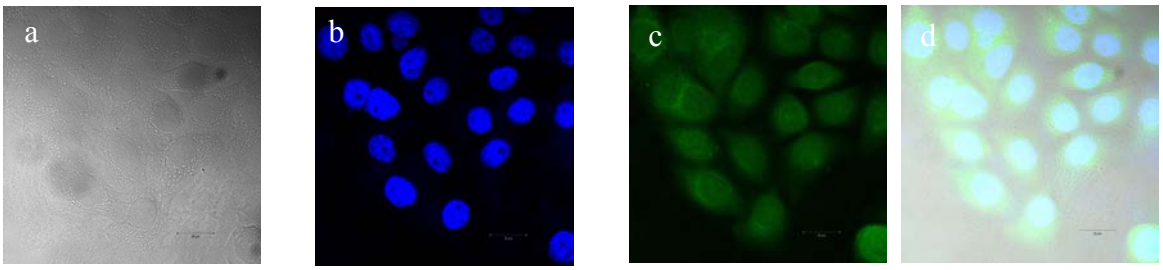

Figure S5.8: 1-photon confocal microscopy images of **4** and DAPI stain in HeLa (fixed cells), (a) DIC image, (b) excitation at 405 nm, emission 420-480 nm (c) excitation at 488 nm, emission >505 nm, (d) overlay of (a)+(b)+(c)

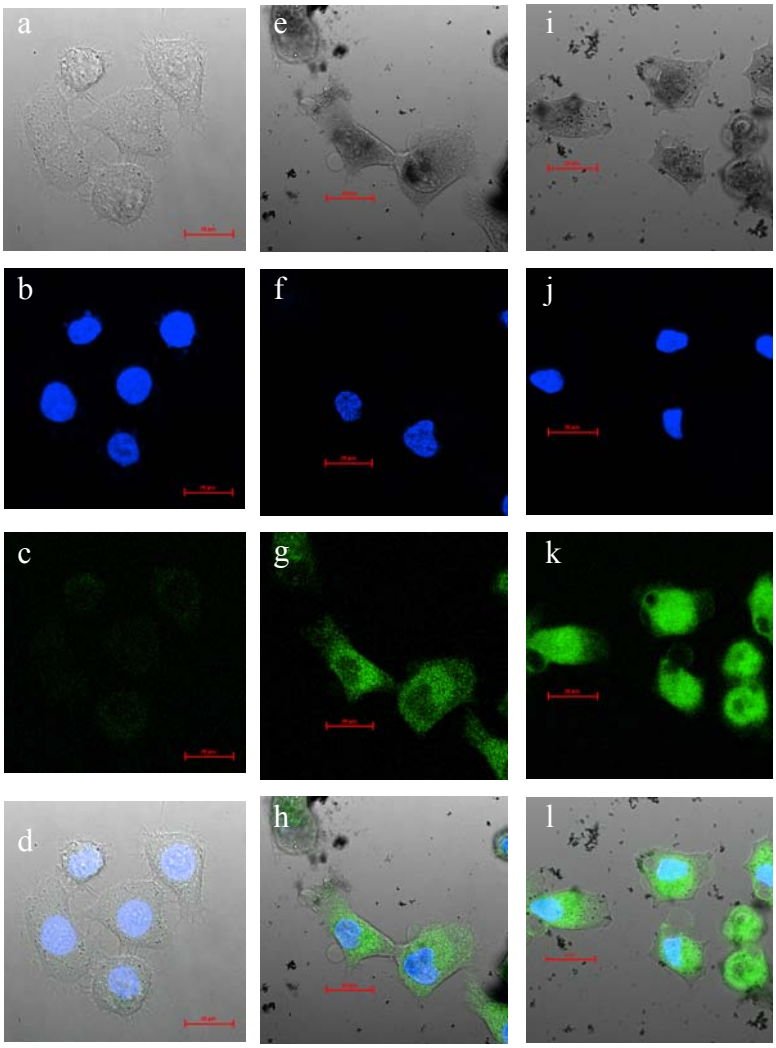

Figure S5.9: (a)-(d):1-Photon confocal microscopy images of 100 μM of **L2**, with 1 h incubation, at 37 °C with Hoechst stain in PC3 cells; (a) brightfield image, (b) excitation at 405nm (nuclear stain, 'blue'), (c) excitation at 488nm (complex, green) , (d) overlay of three previous images; (e)-(h):1-Photon confocal microscopy images of 50 μM of **6**, with 1 h incubation, at 37 °C with Hoechst stain in PC3 cells; (e) brightfield image, (f) excitation at 405nm (nuclear stain, 'blue'), (g) excitation at 488nm (complex, green) , (h) overlay of three previous images; (i)-(l):1-Photon confocal microscopy images of 50 μM of **6**, with 6 h incubation, at 37 °C with Hoechst stain in PC3 cells; (i) brightfield image, (j) excitation at 405nm (nuclear stain, 'blue'), (k) excitation at 488nm (complex, green) , (l) overlay of three previous images.

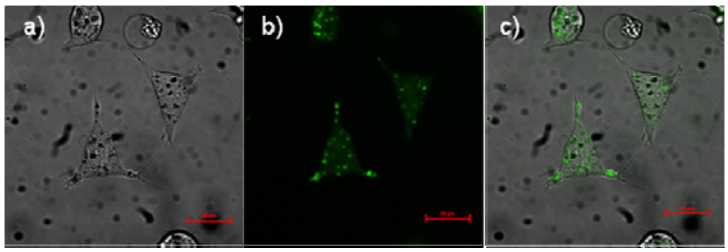

Figure S5.10: 1-photon confocal microscopy images of **4** in HeLa, (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (d) overlay of (a)+(b)

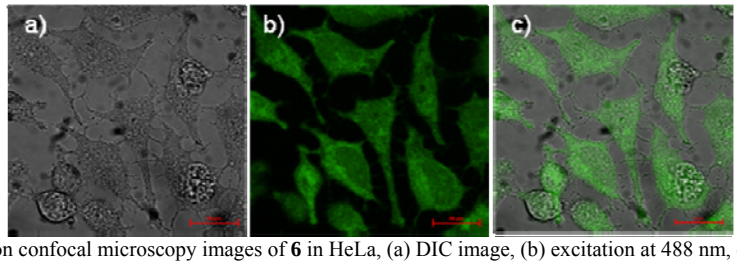

Figure S5.11: 1-photon confocal microscopy images of **6** in HeLa, (a) DIC image, (b) excitation at 488 nm, emission >505 nm, (d) overlay of (a)+(b)

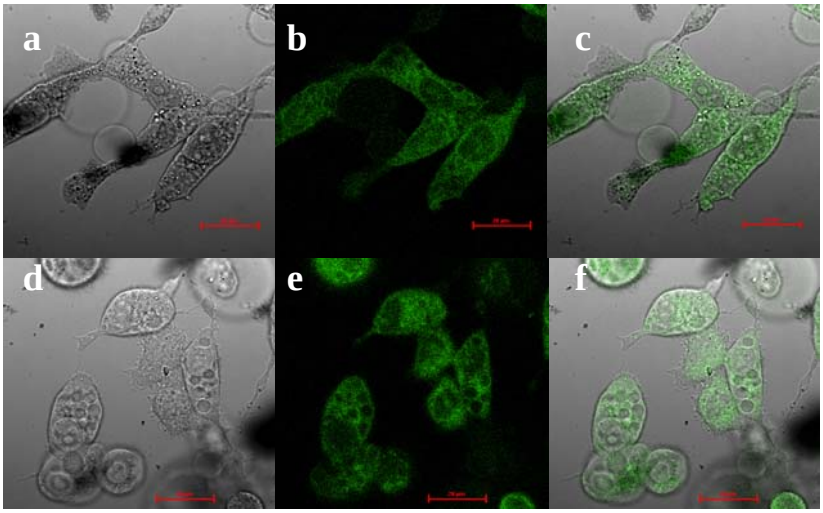

Figure S5. 12 Brightfield and Laser scanning confocal fluorescence microscopy images t 50 μM conc, 1% DMSO: (a-c) Complex **5** in PC-3, incubation time 1h, 37 ºC; (a) DIC image, b) ex 488 nm, emission 516-530 nm, c) overlay (a-b).; (d-f) Complex **6** in PC-3, incubation time 1h, 37 ºC; (d) DIC image, e) ex 488 nm, emission 516-530 nm, f) overlay (d-e).

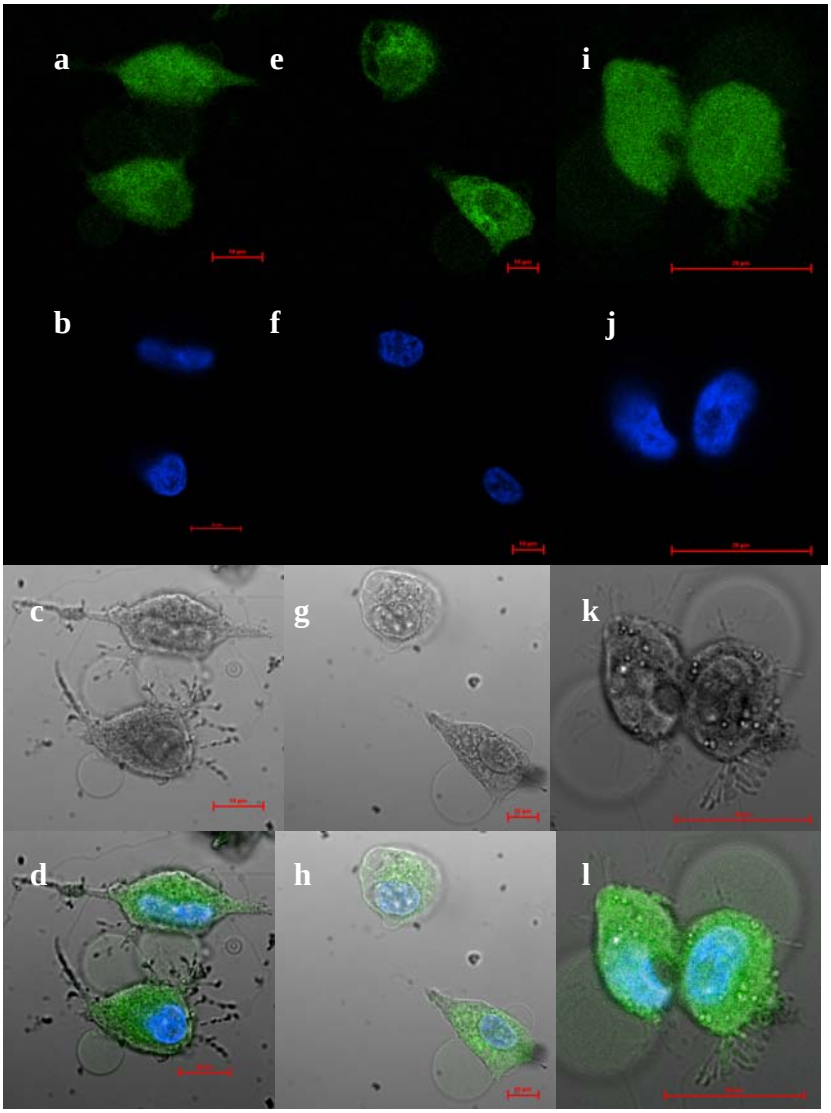

Figure S5.13: (a)-(d):1-Photon confocal microscopy images of 50 µM of **5** after 1 h incubation at 37 ºC with Hoechst stain in HeLa cells; (a) excitation at 488nm (complex, green), (b) excitation at 405nm (nuclear stain, 'blue'), (c) brightfield image , (d) overlay of (a), (b) and (c); (e)-(h):1-Photon confocal microscopy images of 50 µM of **5**, with 1 h incubation, at 37 ºC with Hoechst stain in PC3 cells; (e) excitation at 488nm (complex, green), (f) excitation at 405nm (nuclear stain, 'blue'), (g) brightfield image , (h) overlay of (e), (f) and (g); (i)-(l):1-Photon confocal microscopy images of 50 µM of 6, with 1 h incubation, at 37 ºC with Hoechst stain in HeLa cells; (i) excitation at 488nm (complex, green), (j) excitation at 405nm (nuclear stain, 'blue'), (k) brightfield image , (l) overlay of (i), (j) and (k). After 1 h incubation with **5** or **6** cells show formation of extracellular vacuoles and the occurrence of morphological abnormalities in addition to some nuclear localisation is evident. Scalebar 20 µm.

S6-Fluorescence Lifetime Imaging

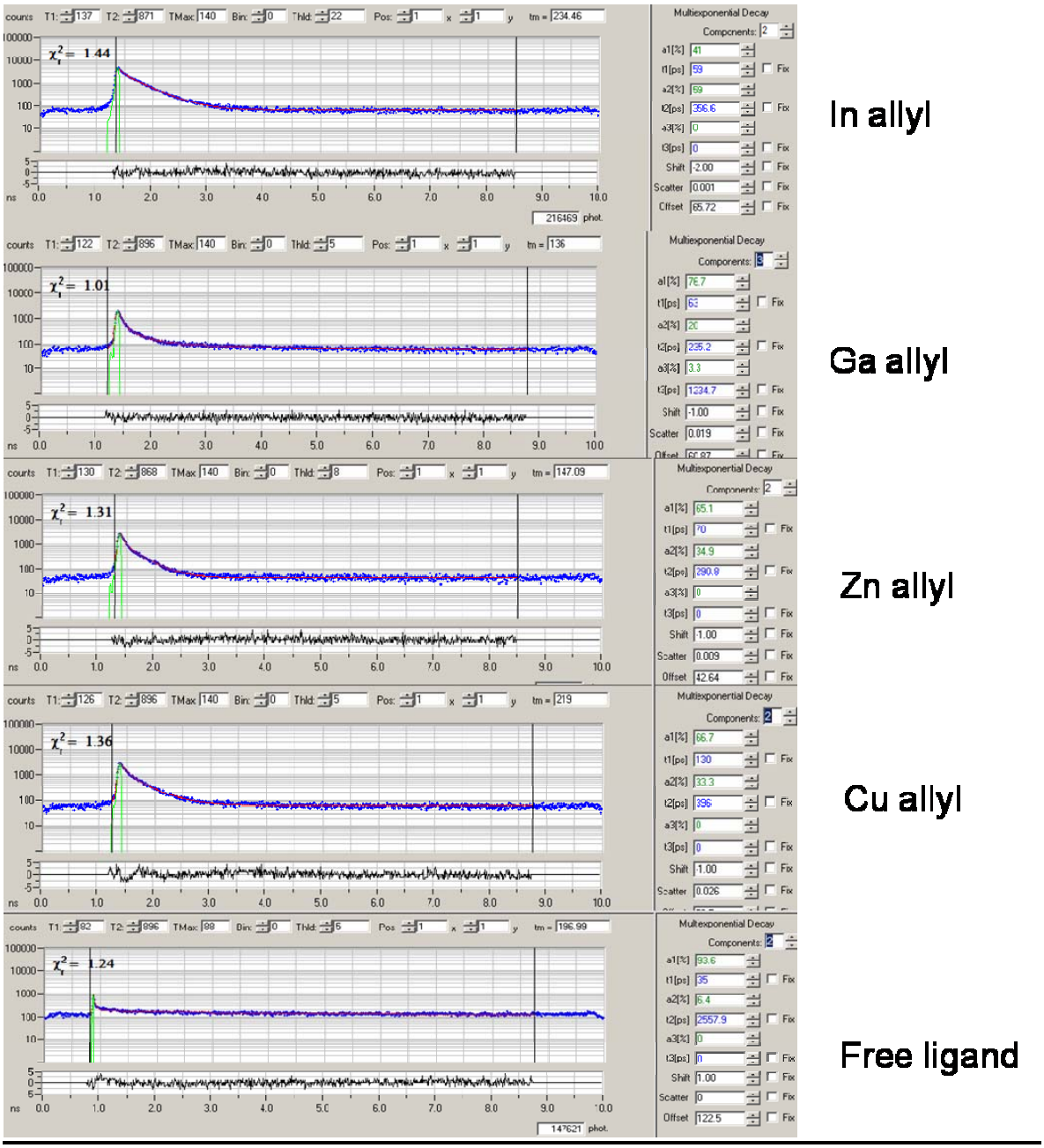

Figure S6.1: Fitted 2 photon excitation (ex 915nm) FLIM decay curves for complexes **4, 6, D, E** and **L3** in DMSO at 50μM

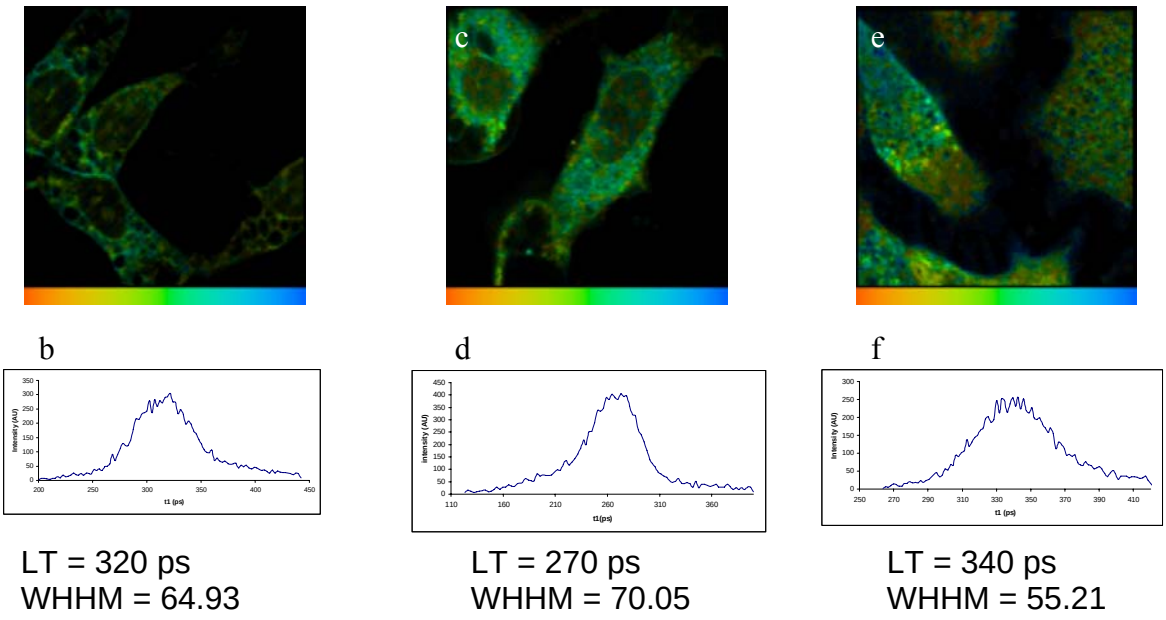

Figure S6.2: a,b) 2-Photon Fluorescence lifetime imaging map for the uptake of **5** at 50µM in PC3 cells after 30 min, with corresponding lifetime distribution plots (ex 910 nm) , where x-axis represents Lifetime / ps and y-axis represents intensity; c,d) 2-Photon Fluorescence lifetime imaging map for the uptake of **5** at 50µM in PC3 cells after 60 min; e,f) 2-Photon Fluorescence lifetime imaging map for the uptake of **5** at 50µM in PC3 cells after 4h

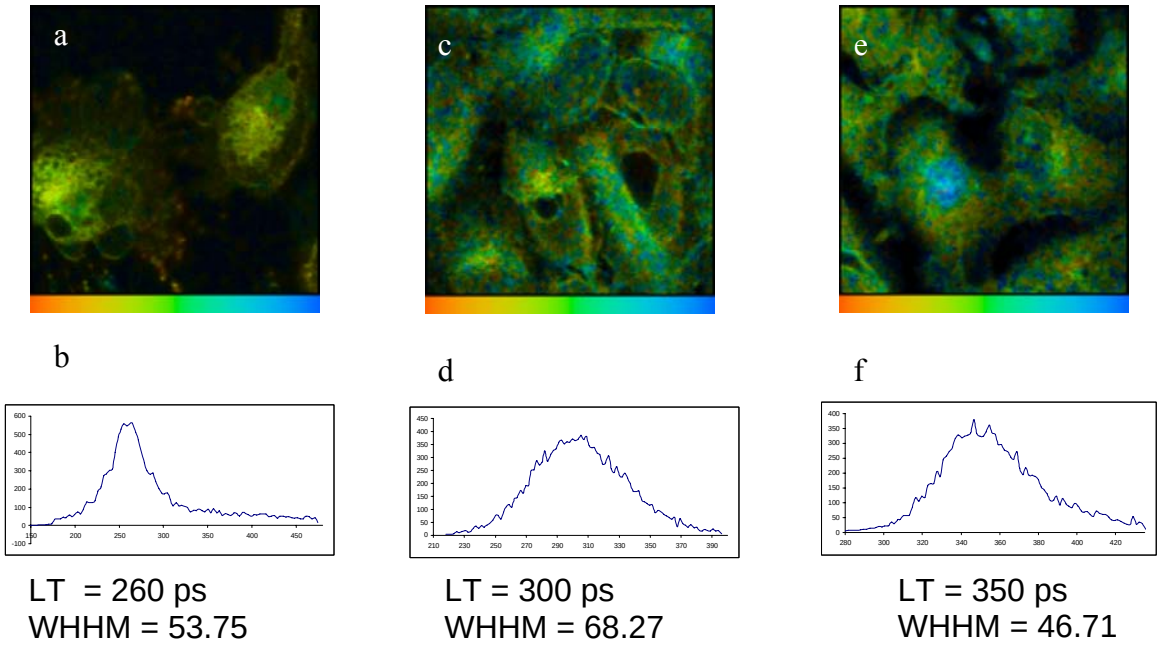

Figure S6.3: a,b) 2-Photon Fluorescence lifetime imaging map for the uptake of **5** at 50µM in HeLa cells after 30 min, with corresponding lifetime distribution plots (ex 910 nm) , where x-axis represents Lifetime / ps and y-axis represents intensity; c,d) 2-Photon Fluorescence lifetime imaging map for the uptake of **5** at 50µM in HeLa cells after 60 min; e,f) 2-Photon Fluorescence lifetime imaging map for the uptake of **5** at 50µM in HeLa cells after 4h

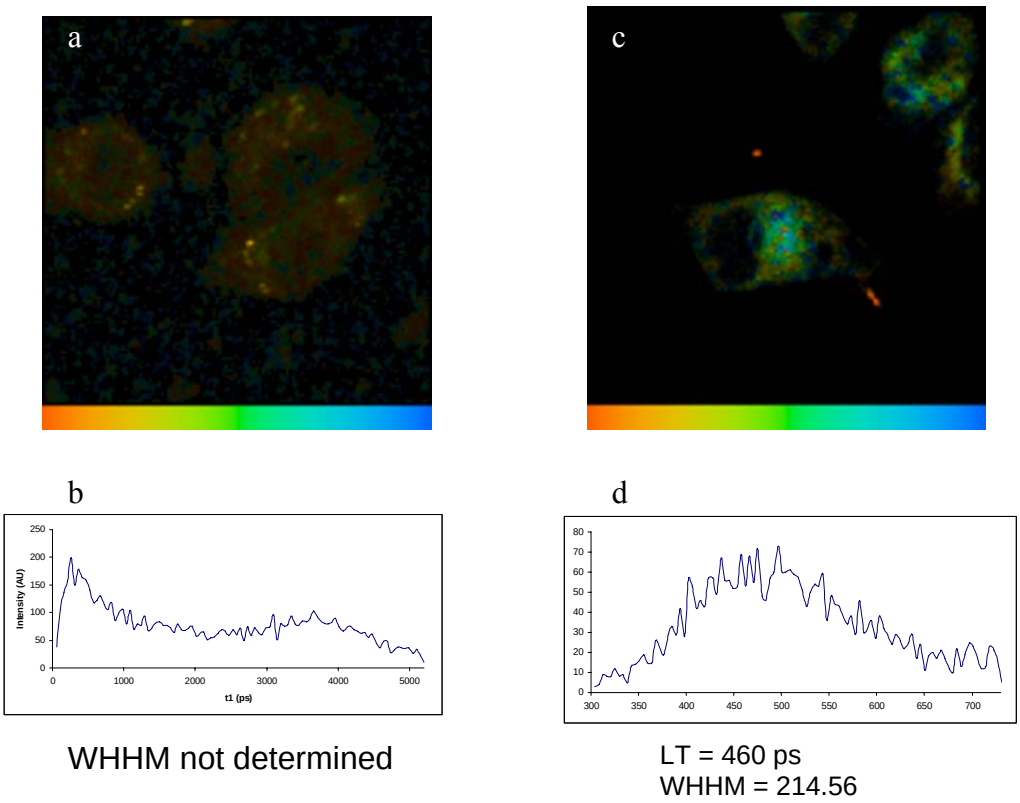

Figure S6.4: a,b) 2-Photon Fluorescence lifetime imaging map for the uptake of **L2** at 100µM in PC3 cells after 4h, with corresponding lifetime distribution plots (ex 910 nm) , where x-axis represents Lifetime / ps and y-axis represents intensity; c,d) 2-Photon Fluorescence lifetime imaging map for the uptake of **3** at 50µM in PC3 cells after 4h.

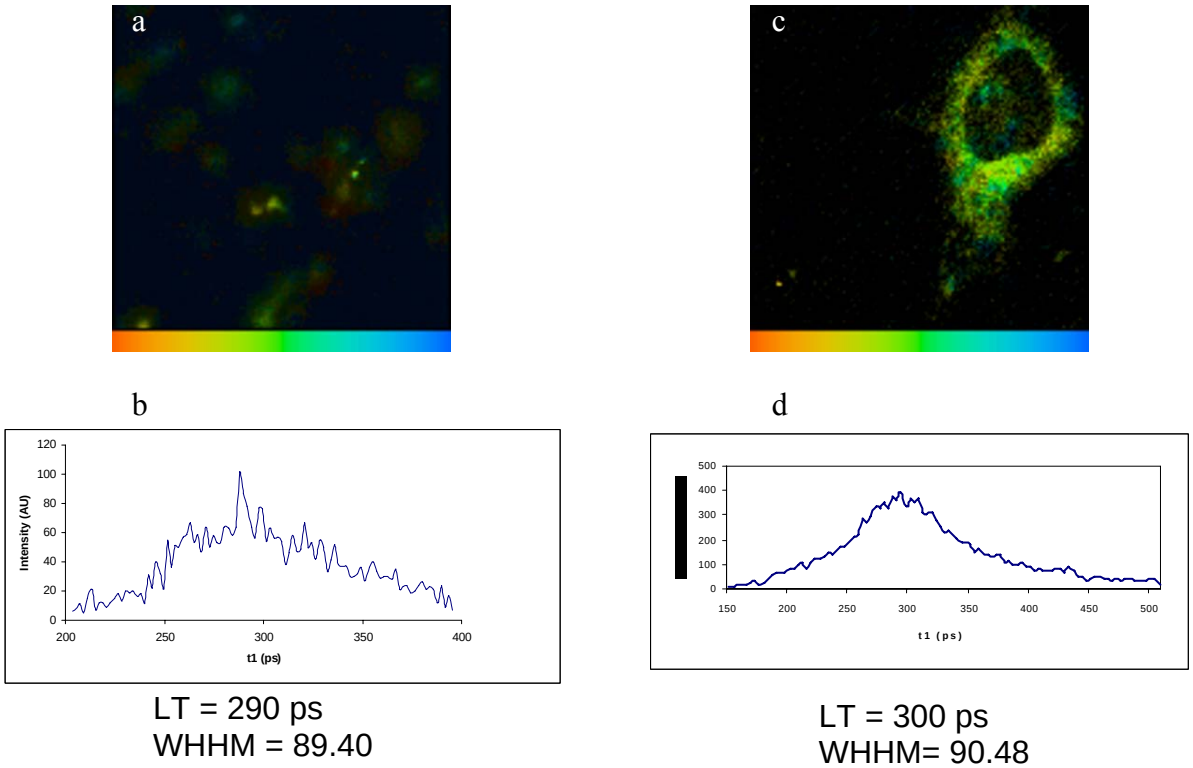

Figure S6.5: a,b) 2-Photon Fluorescence lifetime imaging map for the uptake of **L3** at 100µM in HeLa cells after 1 h, with corresponding lifetime distribution plots (ex 910 nm) , where x-axis represents Lifetime / ps and y-axis represents intensity; c,d) 2-Photon Fluorescence lifetime imaging map for the uptake of **E** at 50uM in HeLa cells after 1 h.

S7-Cytotoxicity study – MI_{50} data

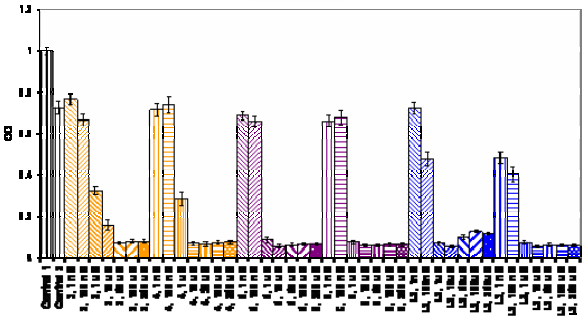

Figure S7.1: Overview of MI_{50} at 48 h for all compounds tested. Where Control 1 = EMEM control (10% FCS) and Control 2 = DMSO control (1:99, DMSO:EMEM containing ca. 10% FCS).

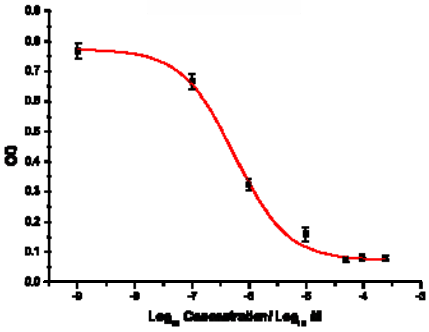

Figure S7.2: Scatter graph representing MI_{50} of **3**

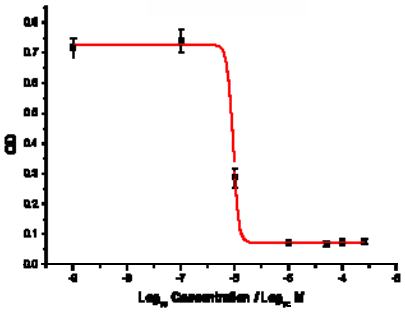

Figure S7.3: Scatter graph representing MI_{50} of **4**

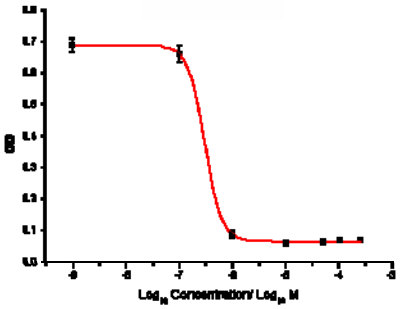

Figure S7.4: Scatter graph representing MI_{50} of **5**

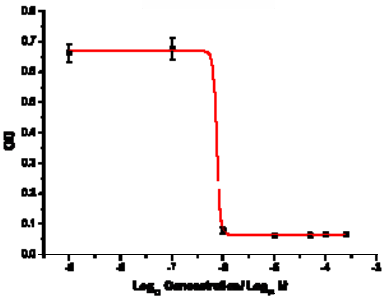

Figure S7.5: Scatter graph representing MI_{50} of **6**

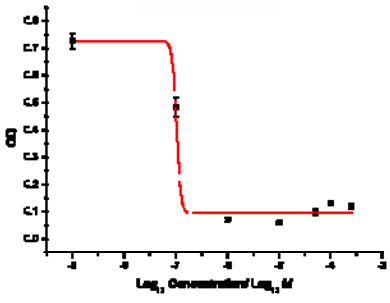

Figure S7.6: Scatter graph representing MI_{50} of **L2**

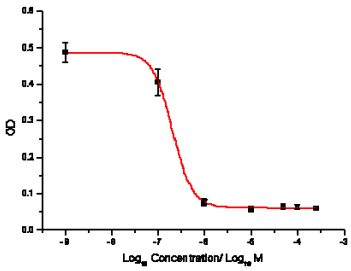

Figure S7.7: Scatter graph representing MI_{50} of **L3**

To further assess the behaviour of complexes **3-6** over the time scale of a typical cell uptake experiment, an MTT assay was performed using 10 μM concentrations of compounds in FCS-free medium and 1% DMSO. For the time-dependent experiment, HeLa cells were incubated with the complexes or corresponding free proligands for 1h, 3h, 6h and 24h and cell viability was estimated. MTT assays showed that the compounds display *rather low* toxicity after 1h at a 10μM concentration. The time lapse experiment shows that at this concentration and at 24 h time point Cu(ATSM) is essentially the most cytotoxic in the series. The gallium and indium bis(thiosemicarbazonato) complexes **3-6** were of relatively low to moderate toxicity up to 6h (MI_{50} > ca. 10 μM), but their toxicity increased significantly after 24h.

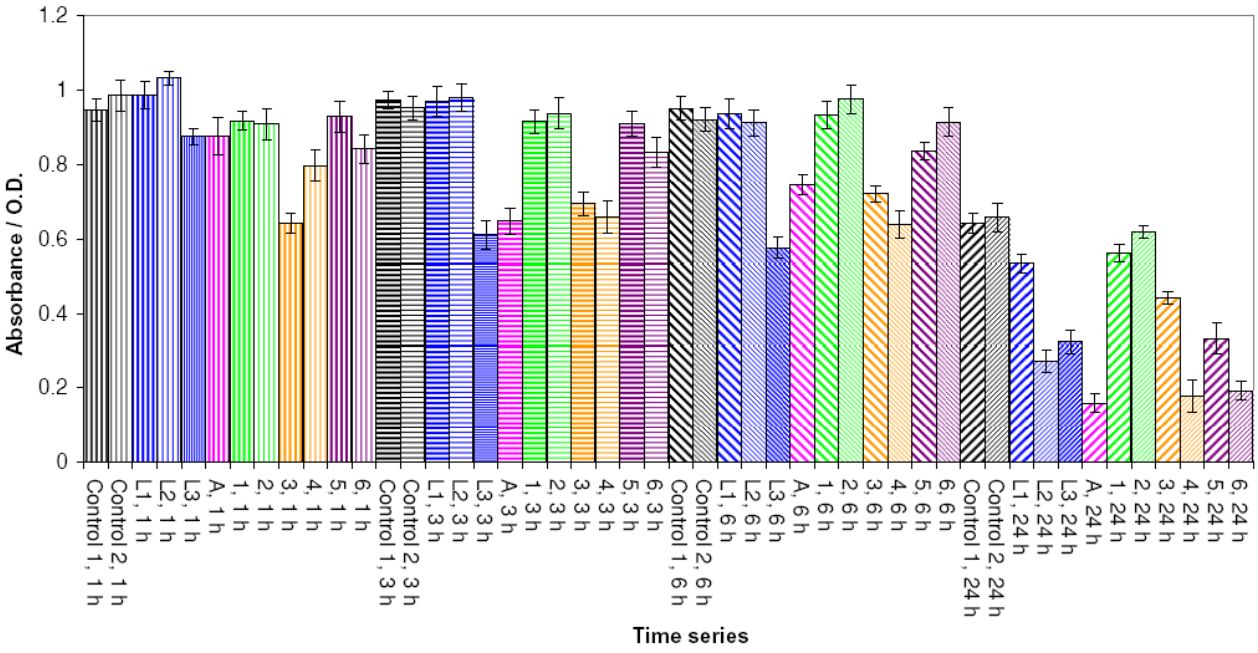

Figure S7.8: Overview of timelapse MTT assay in HeLa at 1, 3, 6 and 24h h for all compounds tested. Where Control 1 = serum free EMEM control and Control 2 = serum free EMEM control containg 1% DMSO (1:99, DMSO:EMEM).

MTT Control experiments and comparison of cells viability after treatment with compounds A, D and 4:

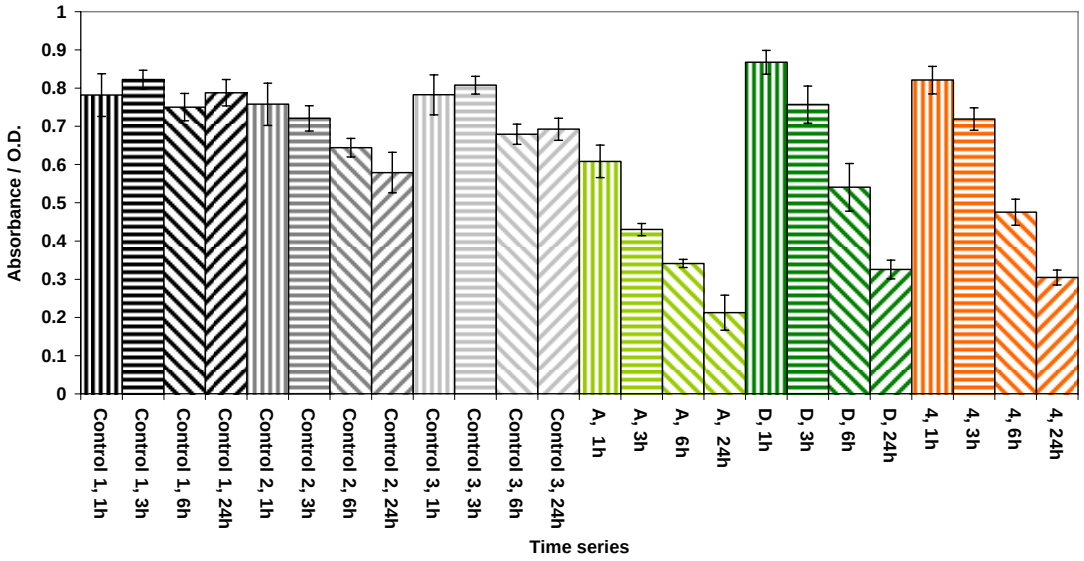

Figure S7. 9 Overview of a time-course MTT assays to verify HeLa cells viability under conditions comparable to those of imaging experiments at 1, 3, 6 and 24 h:
Control 1 = EMEM containing 10% foetal calf serum (FCS);
Control 2 = serum free EMEM;
Control 3 = serum free EMEM containing 1% DMSO;

Compound A: known Zn(ATSM) (10 μM in serum free EMEM containing 1% DMSO);
Compound D: Zn(II) Allyl-substituted compound (10 μM in serum free EMEM containing 1% DMSO);
Compound 4: Ga(III) Allyl substituted compound (10 μM in serum free EMEM containing 1% DMSO).