Supporting Information

Tailoring the properties of a hypoxia-responsive 1,8-naphthalimide for imaging applications

Kylie Yang[1], Kathryn G. Leslie[1], Sally Y. Kim[2], Bill Kalionis[3], Wojciech Chrzanowski[2,4], Katrina A. Jolliffe[1]*, Elizabeth J. New[1,4]*

1. School of Chemistry, The University of Sydney, NSW, 2006 Australia
2. Faculty of Pharmacy, The University of Sydney, NSW, 2006, Australia
3. Department of Maternal-Foetal Medicine Pregnancy Research Centre and University of Melbourne. Department of Obstetrics and Gynaecology, Royal Women's Hospital, Parkville, VIC, 3052, Australia
4. The University of Sydney Nano Institute, NSW 2006, Australia
kate.jolliffe@sydney.edu.au; elizabeth.new@sydney.edu.au

Table of Contents

Supplementary Experimental Methods

Synthetic methods

Synthesis of NpHy1 (*N*-Butyl-4-nitro-1,8-naphthalimide).

Butylamine (0.22 mL, 2.3 mmol) was added to a suspension of **1** (0.50 g, 2.1 mmol) in dioxane (20 mL), and the reaction was heated at reflux for 16 h. The reaction mixture was then poured onto ice water, and the precipitate collected by filtration and washed with water to give **NpHy** as a light brown solid (0.40 g, 66%). **M.P.** 104-106°C, (lit.[1] 103-104°C); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.84 (d, *J* = 8.6, 1H), 8.74 (d, *J* = 7.3, 1H), 8.69 (d, *J* = 8.0, 1H), 8.40 (d, *J* = 8.0, 1H), 7.98 (dd, *J* = 8.6, 7.3, 1H), 4.19 (t, *J* = 7.6, 2H), 1.78-1.68 (m, 2H), 1.52-1.39 (m, 2H), 0.99 (t, *J* = 7.3, 3H); **APCI-MS** $(M + H)^+$ 299.

N-Butyl-4,5-dinitro-1,8-naphthalimide (2).

Synthesis was adapted from a published procedure.[2] Conc. nitric acid (3.0 mL) was added dropwise to a suspension of **NpHy** (0.10 g, 0.34 mmol) in conc. sulfuric acid (3.0 mL) at 0 °C. The reaction was warmed to room temperature and stirred for 3 h. The reaction mixture was poured onto ice and the precipitate collected by filtration to give **2** as a pale brown solid (97 mg, 84%). **M.P.** 322-326 °C, (lit.[3] 325-327 °C); **^{1}H NMR** (200 MHz, $CDCl_3$): δ 8.81 (d, *J* = 8.1, 2H), 8.44 (d, *J* = 8.1, 2H), 4.20 (t, *J* = 7.4, 2H), 1.80-1.65 (m, 2H), 1.54-1.35 (m, 2H), 0.98 (t, *J* = 7.2, 3H); **APCI-MS** $(M + H)^+$ 344.

***N*-Butyl-4-butylamino-5-nitro-1,8-naphthalimide (NpHy2)**. Synthesis was adapted from a published procedure.[3] Butylamine (30 μL, 0.29 mmol) was added to a solution of **2** (90 mg, 0.26 mmol) in ethanol (10 mL) and heated to reflux for 16 h. The solvent was evaporated and the crude residue purified by silica flash chromatography (10 % ethyl acetate, 90 % hexane) to give **NpHy1** as a red solid (0.045 g, 46 %). **M.P.** 115-117 °C, (lit.[3] 124-125 °C); **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.58 (d, *J* = 7.9, 1H), 8.52 (d, *J* = 8.6, 1H), 7.63 (d, *J* = 7.9, 1H), 6.90 (d, *J* = 8.6, 1H), 5.11 (s, 1H), 4.14 (t, *J* =7.5, 2H), 3.31-3.25 (m, 2H), 1.76-1.64 (m, 4H) 1.54-1.37 (m, 4H), 1.02-0.94 (m, 6H); **APCI-MS** $(M + H)^+$ 370.

Biological studies

Cytotoxicity

DLD-1 cells were seeded at 10,000 per well for a (4 h experiment) and 4,000 per well (for a 50 h experiment). They were left to adhere overnight, then a solution of probe in media was added to in quadruplicate. The cells were incubated for either 4 h or 50 h, then an alamarBlue® assay was carried out according to the manufacturer's protocol.

Nikon A1 R live cell imaging

DLD-1 cells were seeded at 100,000 cells per MatTek dish and allowed to adhere overnight. The media was removed, and the cells were rinsed with thrice with PBS then added 0.5 mL FluoroBrite™ media supplemented with 1% glutamine and 2% FBS. The hypoxic stage incubator

and time course experiment was set up, then 0.5 mL 2× probe solution in FluoroBrite™ was added to the dish and the experiment started immediately afterwards. The cells were then imaged over 20 min using a spectral scanner and 403 nm laser excitation.

Stem cell experiments

Transduced foetal chorionic and maternal mesenchymal stromal cells were used as preferred over primary human placenta cells, for reproducibility of responses to the probes. CMSC29 and DMSC23 cell lines, were cultured in AmnioMAX™ with its supplement (Life Technologies) and Mesencult™ proliferation kit (STEMCELL Technologies) respectively, both supplemented with 1% penicillin-streptomycin and 1% GlutaMAX™ (Life Technologies).[4] Cells were used at passages 25-26. Cells were seeded at 4,000 cells per well on a 96-well plate then incubated overnight in normal culture conditions, at 37°C and 5% CO_2.

A 1,000× solution of probe in DMSO was quickly mixed into FluoroBrite™ DMEM media (ThermoFisher Scientific) supplemented with 1% glutamine and 10% FBS to make a 1× solution. CMSC29 and DMSC23 cells were washed with FluoroBrite DMEM media to get rid of phenol red, then dosed with the 1× probe solutions. Two plates of cells were prepared and one plate was placed on an IncuCyte® live-cell analysis system under normoxia (19 % oxygen), while one plate in an IncuCyte® system placed inside Don Whitley H135 HEPA Hypoxystation (Don Whitley Scientific) under hypoxia (1% oxygen) with 94% N_2 and 5% CO_2. The cells were imaged every 2 h simultaneously for phase and fluorescent images. The IncuCyte® was fitted with a 440 - 480 nm excitation passband and a 504 - 544 nm emission passband. The mean intensity of the green channel was obtained and the intensity of the first data point was used to normalise the data between hypoxia and normoxia for comparison.

Uptake experiments

150,000 DLD-1 cells were seeded in a poly-D-lysine coated MatTek dish and allowed to adhere overnight. Afterwards, probe solutions in media were prepared by firstly dissolving the probe in DMSO then quickly adding it to the media. The cells were then incubated in the probe solution for 20 minutes. Afterwards, the cells were washed with PBS and put in FluoroBrite™ media. They were imaged in an Olympus FV1000 microscope using a 60× water objective and 405 nm laser excitation.

Leica SP5 live spheroid imaging

DLD-1 cells were trypsinised after they were 50% confluent and mixed with a pipette to break up clumps. 20,000, 10,000 and 5,000 DLD-1 colon cancer cells were seeded into a sterile 96-well plate (Costar® ultra-low cluster, ultra-low attachment, polystyrene, non-polystyrene) and were grown in 100 µl of 2 % media over 3 days. The media was then removed and solutions of probe in media were added overnight. The spheroids were collected with a pipette with a trimmed tip and added to a microcentrifuge tube. They were washed 3 times with PBS and then imaged in FluoroBrite™ media using a Leica SP5 microscope with a 100× water objective and 810 nm 2 photon excitation. Fluorescence was collected between 515 and 600 nm and image analysis was performed on Fiji / Image J software.

Supplementary Data

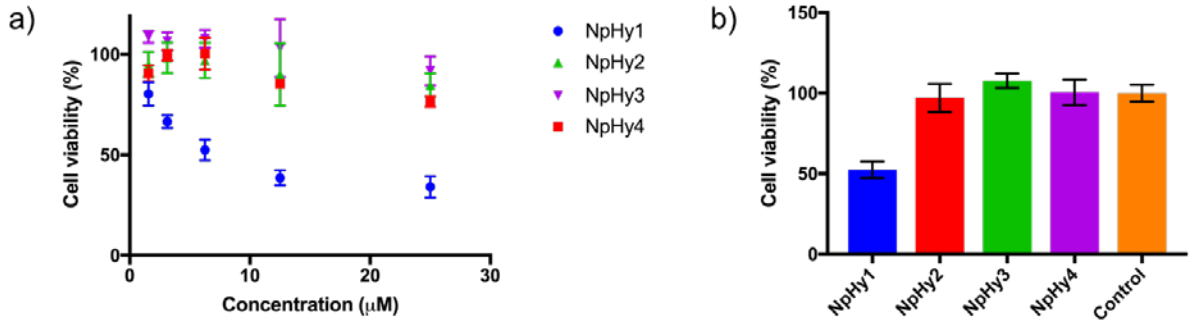


Figure S1: (a) Cell viability of DLD-1 cells at 4 h after treatment with each of the NpHy analogues at varying concentrations (b) Cell viability of DLD-1 cells at 4 h after treatment with each of the NpHy analogues at 5 μM.

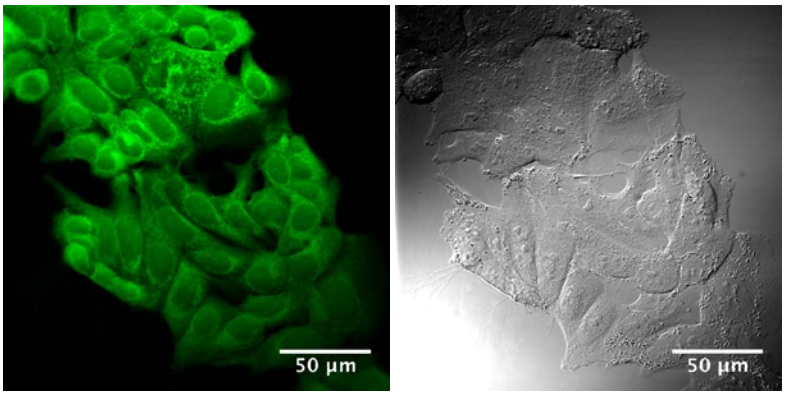


Figure S2: Confocal microscopy image of DLD-1 cells treated with **NpHy1** (20 min, 5 μM) under normoxia. Left: excitation at 405 nm, emission collected 500 – 600 nm. Right: brightfield image

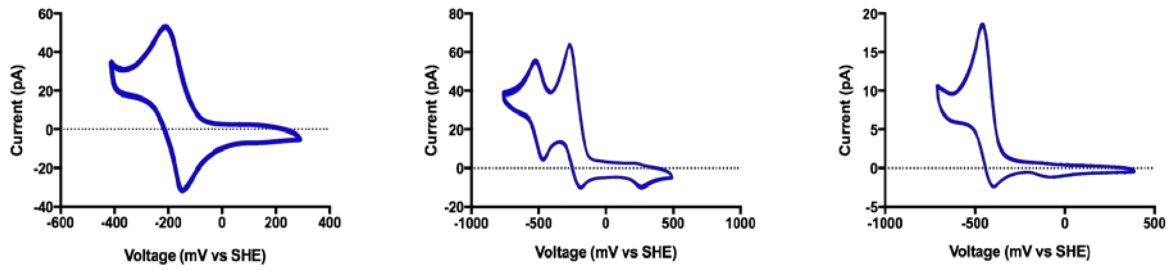


Figure S3: Cyclic voltammagrams for **NpHy1**, **NpHy2**, and **NpHy3** (1 mM, with 0.1 M tetrabutylammonium hexafluorophosphate in MeCN).

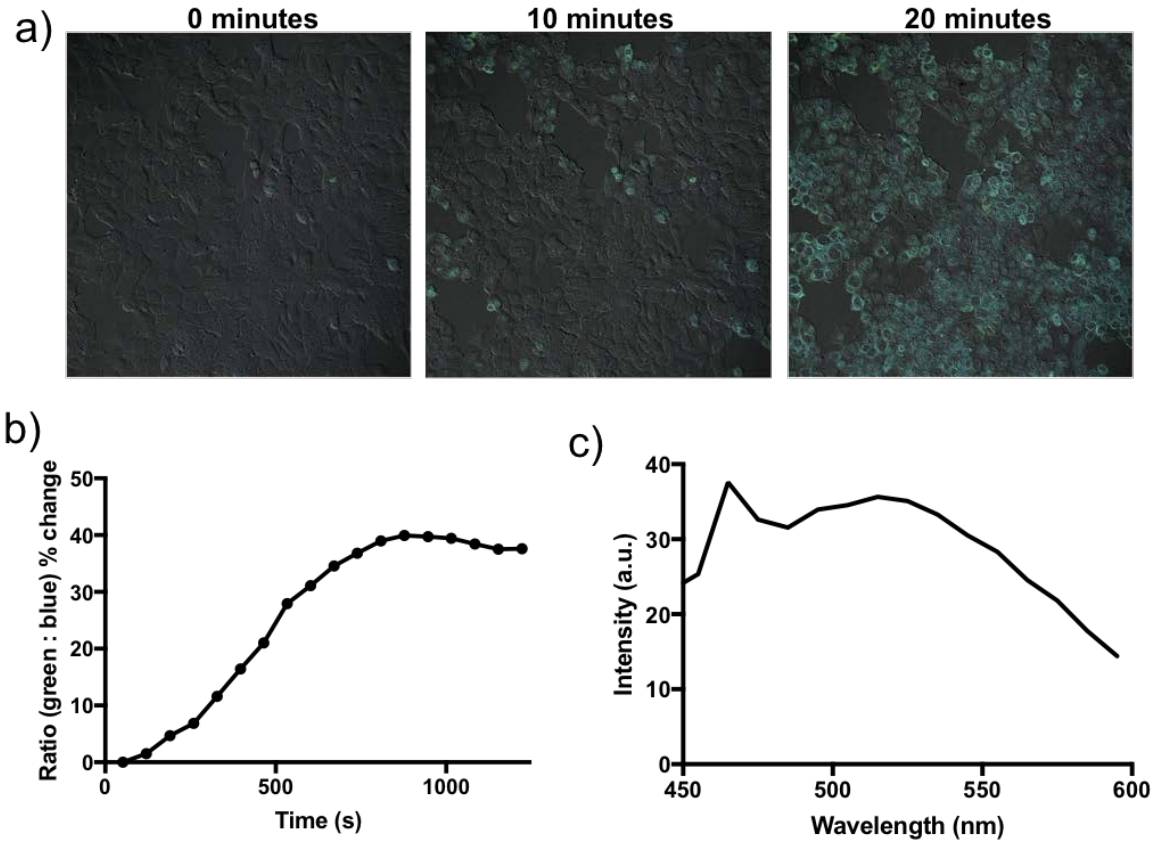


Figure S4: a) Confocal fluorescence microscopy of DLD-1 cells incubated under 1% oxygen with 5μM **NpHy2**. b) Percentage increase in the green (495 - 595 nm): blue (425 - 485 nm) fluorescence ratio over time. c) Spectral scan of **NpHy2** in cells after 10 min.

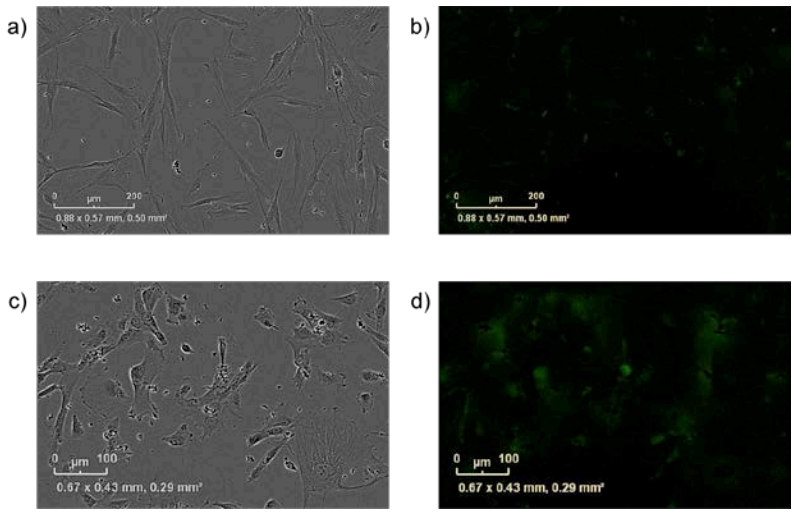


Figure S5: Brightfield images of (a) DMSC23 and (c) CMSC29 cells, and fluorescence microscopy images of (b) DMSC23 and (d) CMSC29 cells incubated with **NpHy3** after 4 h under hypoxic conditions (1% oxygen).

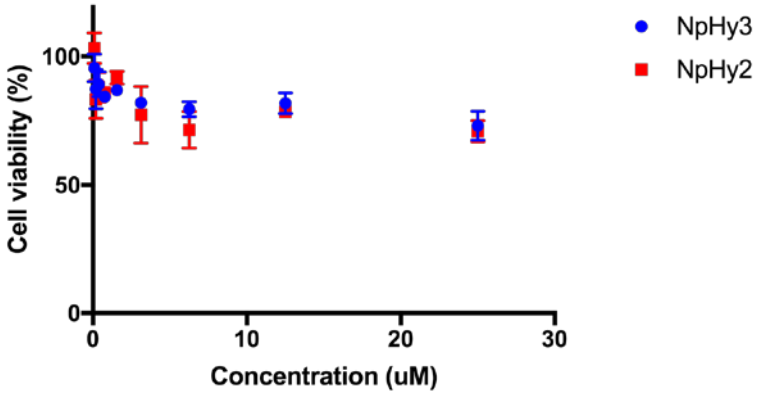


Figure S6: 50 h cell viability of DLD-1 cells treated with each of the **NpHy3** and **NpHy4** at varying concentrations

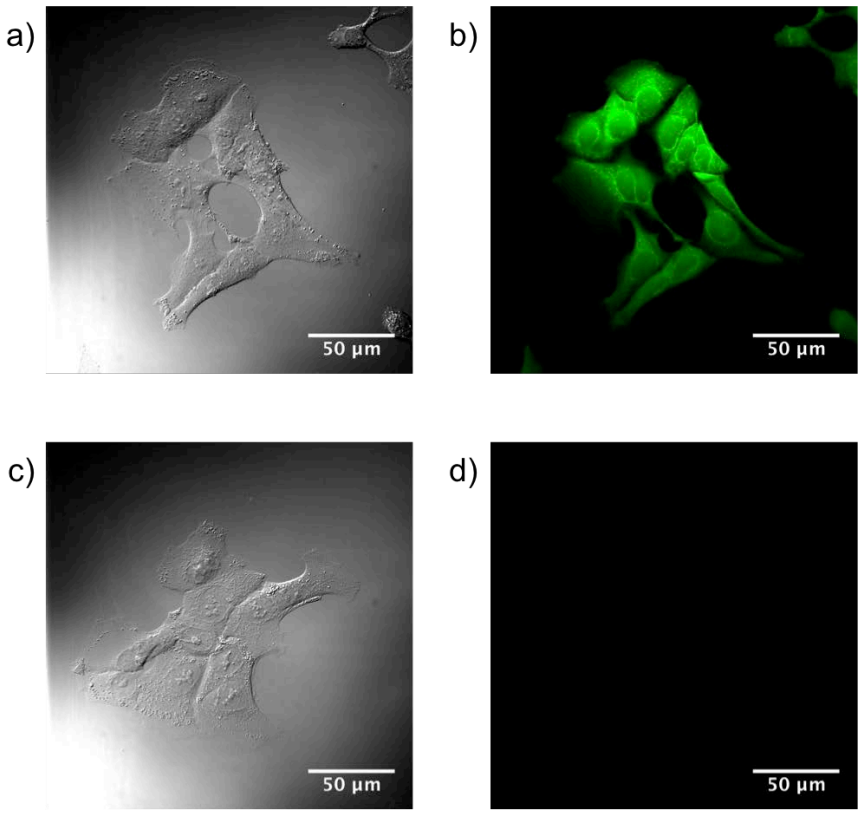
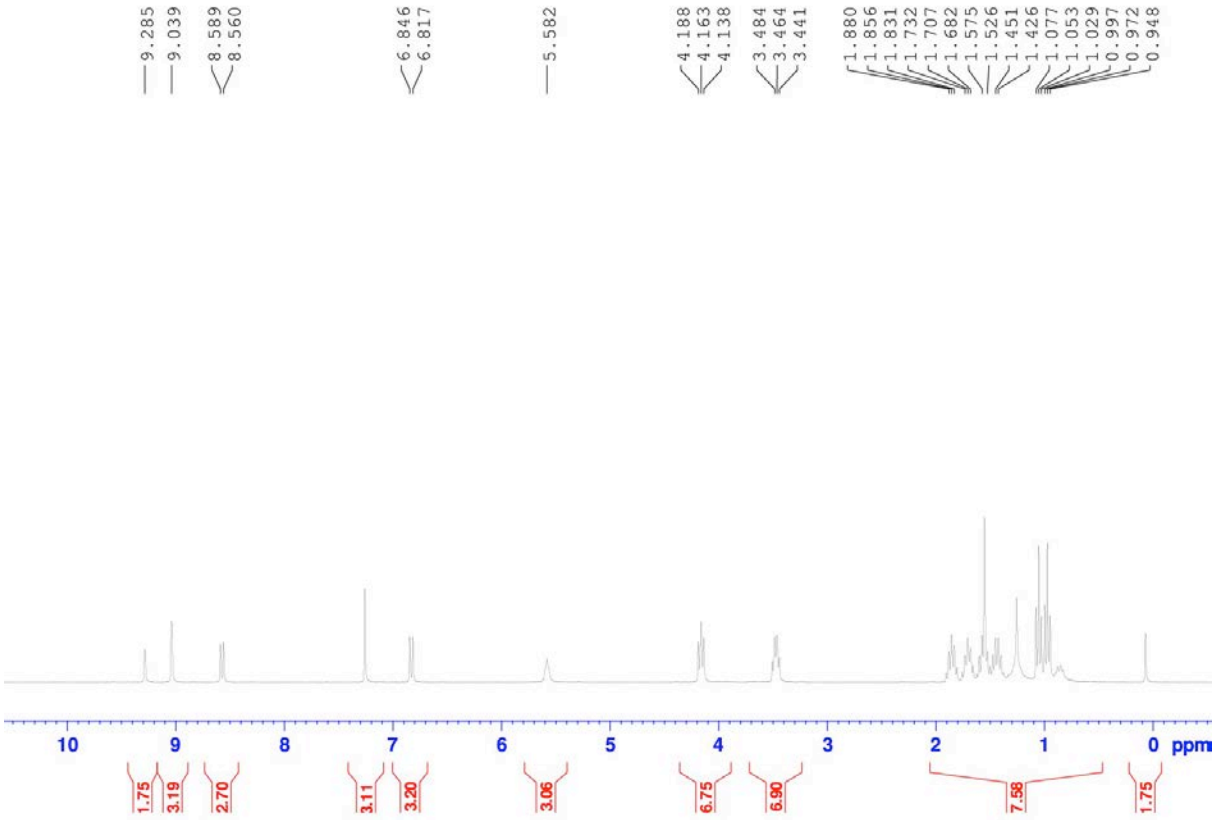
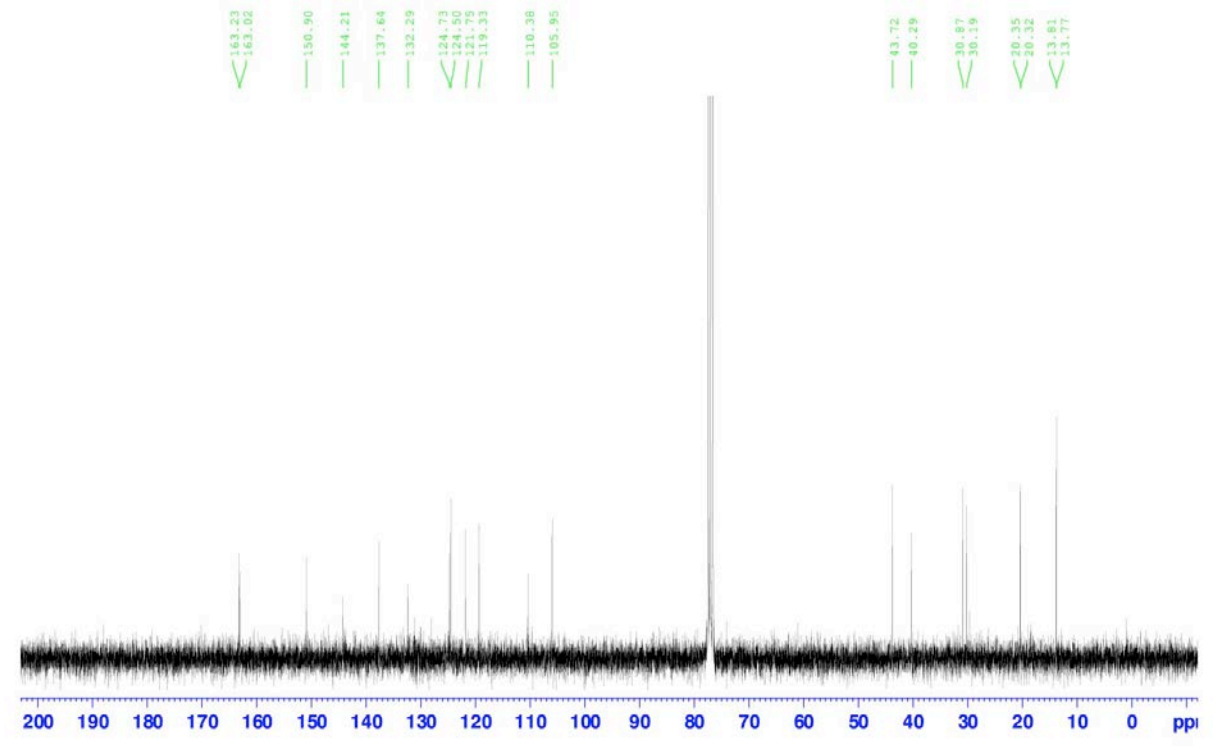


Figure S7: DLD1 cells treated with 5 uM **NpHy1** (a) brightfield (b) fluorescence or **NpHy4** (c) brightfield (d) fluorescence for 20 min and imaged on an Olympus FV 1000 with a 405 nm laser.

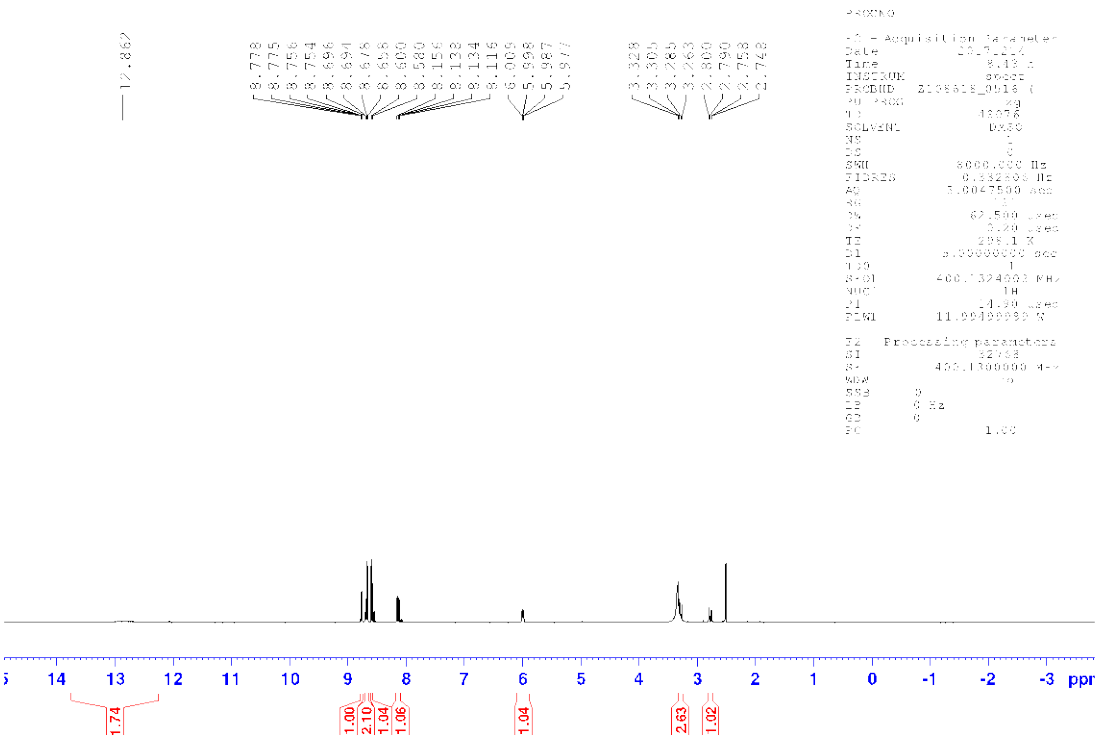
Spectra of novel compounds



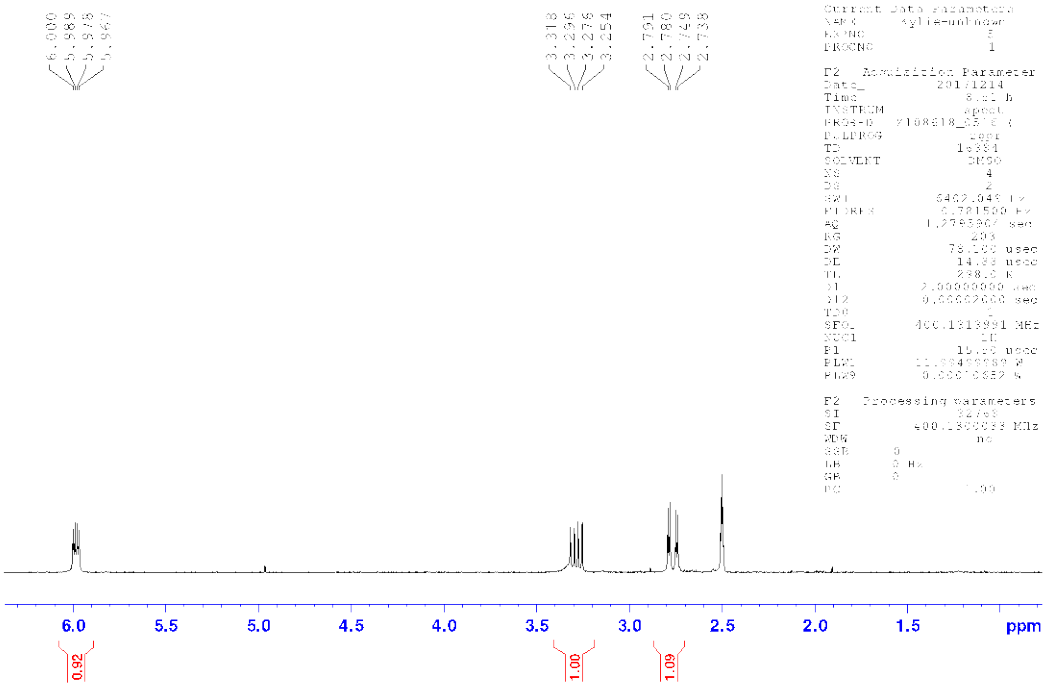
NpHy3, ^{1}H NMR, 300 MHz, $CDCl_3$



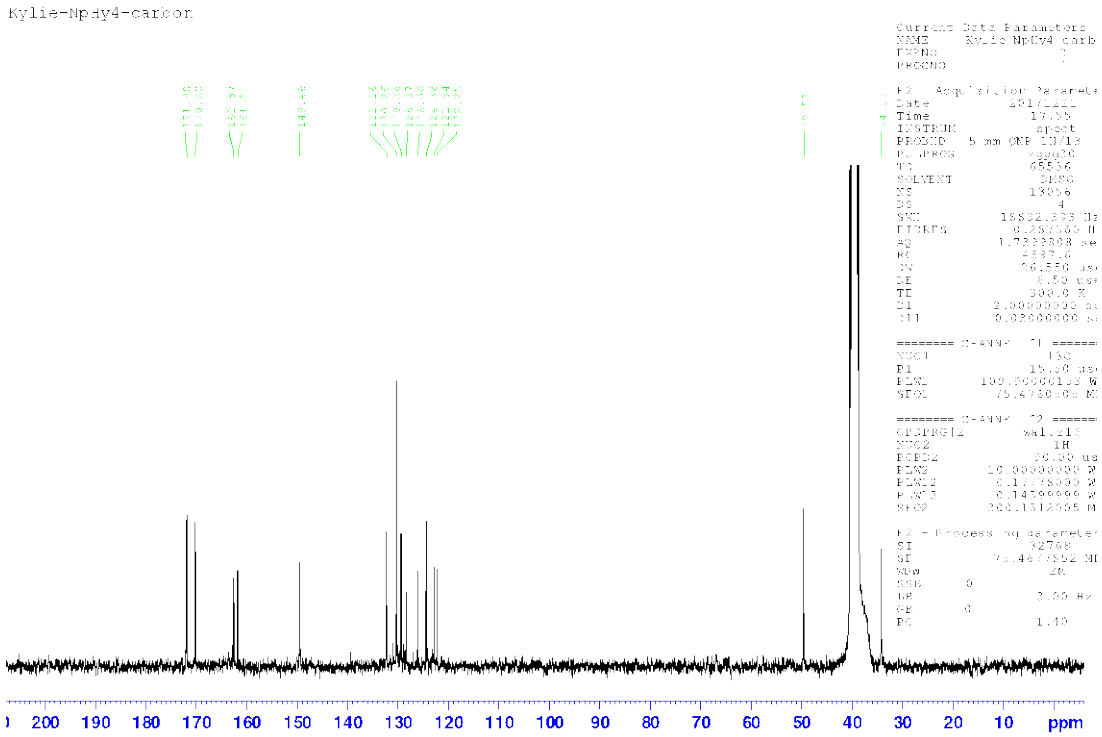
NpHy3, ^{13}C NMR, 75 MHz, $CDCl_3$



NpHy4, ^{1}H NMR, 400 MHz, DMSO-d_6



NpHy4, ^{1}H NMR, 400 MHz, DMSO-d_6, water suppression, alkyl region



NpHy4, ^{13}C NMR, 75 MHz, DMSO-d_6

References

1. E. R. Triboni, P. B. Filho, R. G. de Souza Berlinck and M. J. Politi, *Synthetic Commun.*, 2004, **34**, 1989-1999.
2. M. Zhu, J. Zhang, G. Yu, H. Chen, J. Huang and Y. Liu, *Chem.-Asian J.*, 2012, **7**, 2208-2212.
3. M. Alexiou and J. Tyman, *J. Chem. Res.*, 2001, **2001**, 59-61.