Supplementary Information for:

Mitochondrially targeted redox probe reveals the variations in oxidative capacity of the haematopoietic cells

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GENERAL EXPERIMENTAL METHODS

All solvents used were laboratory grade and were dried over appropriate drying agents when required. MilliQ water was used to prepare all aqueous solutions. Merck 230-400 mesh Kieselgel 60 was used for column chromatography and Merck Kieselgel 60 0.25 mm F254 precoated sheets were used for analytical thin layer chromatography. Chemicals were obtained from Sigma-Aldrich, Alfa Aesar and Combi Blocks and used as received.

SPECTROSCOPIC METHODS

$^1$H NMR spectra were collected in commercially available deuterated solvents (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker DRX 300 or Bruker Ascend 400 and 500 spectrometer at the School of Chemistry NMR Facility at the University of Sydney. $^{13}$C NMR were recorded on Bruker Ascend 400 and 500 spectrometers at frequencies of 100 MHz and 125 MHZ. All chemical shifts are reported in the standard δ notation of parts per million using the peak of the residual solvent proton signals as an internal reference. $J$ values are given in Hertz. Low-resolution mass-spectral analyses were performed by liquid chromatography-mass spectrometry (Finnigan LCQ quadrupole ion trap mass spectrometer). Spectroscopic measurements were performed in 100 mM HEPES, pH 7.4. Fluorescence measurements were collected using a bench top Varian Cary Eclipse fluorimeter and Perkin Elmer Enspire Multimode Plate Reader at 10 nm slit widths.

QUANTUM YIELDS

The quantum yield ($\Phi$) of the NpFR2 was calculated using fluorescein as a reference. For calculation of quantum yield, five concentrations of the probe (in HEPES buffer) and fluorescein standards in (0.1 M NaOH) were prepared. Their fluorescence spectra were recorded at same excitation of 488 nm. The integrated fluorescence intensities (excited at 488 nm) were plotted against the absorbance (at 488 nm) for both the standards and the probe. The quantum yield was calculated using the below equation

$$\Phi_x = \Phi_S (D_x / D_S)$$

Where $\Phi$ is the quantum yield, $D$ is slope, $S$ and $X$ represents the standard and the sample respectively.
SYNTHESIS OF NpFR2

(3-Aminopropyl)triphenylphosphonium bromide hydrobromide (2)
3-Bromopropylamine hydrobromide (1.00g, 3.82 mmol) and triphenylphosphine (0.838 g, 3.82 mmol) were added to 5 mL of acetonitrile and the resulting suspension was heated to reflux for 12 h. The reaction mixture was cooled to room temperature and hexane (15 mL) was added. The resulting solid was dissolved in isopropanol (100 mL), a minimal amount of diethyl ether (30 mL) was added and the solution left overnight in the refrigerator to give fine colourless crystals (0.860 g, 47%). $^1$H NMR (400 MHz, DMSO-d$_6$): δ 7.9 (t, J=6.8, 3H), 7.8 – 7.75 (m, 15H) 3.74 – 3.71 (m, 2H), 2.93 (t, J=7.6, 2H), 1.90 – 1.87 (m, 2H) ppm.

(3-(6-Bromo-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)propyl)triphenylphosphonium (4)
To a solution of 4-bromo-1,8-naphthalic anhydride (3.0 g, 10.8 mmol) in EtOH (100 mL) was added 2 (3.7g, 11.8 mmol). The mixture was heated to reflux for 17 hours before the solvent was removed under vacuum and the product recrystallised in EtOAc to give yellow crystals of 4 (4.36 g, 7.42 mmol, 70%). $^1$H NMR (400 MHz, DMSO-d$_6$): δ 8.62 (d, J = 8.4, 1H), 8.56 (d, J = 8.5, 1H), 8.40 (d, J = 7.9, 1H), 8.35 (d, J = 7.8, 1H), 8.04 (t, J = 7.6, 1H), 7.5 – 7.45 (m, 15H), 4.27-4.25 (m, 2H), 3.24-3.21 (m, 2H), 1.88-1.85 (m, 2H) ppm.
(3-(6-bromo-5-nitro-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl)propyl)triphenylphosphonium (5)

To a solution of 4 (750 mg, 1.26 mmol) in sulfuric acid (10 mL), sodium nitrate (0.11 g, 1.3 mmol) was added and the solution was stirred for 1 h at -10 °C. The mixture was added slowly to ice-water (50 mL), and the resulting suspension filtered, dried and subjected to purification by flash chromatography (hexane : EtOAc; 2:1) to give 5 as a pale yellow solid (512 mg, 0.82 mmol, 36%). 1H NMR (400 MHz, DMSO-d6): δ 8.64 (d, J = 8.4, 1H), 8.59 (d, J = 8.5, 1H), 8.45 (d, J = 7.9, 1H), 8.1 (t, J = 7.6, 1H), 7.5 – 7.45 (m, 15H), 4.26-4.24 (m, 2H), 3.24-3.22 (m, 2H), 1.87-1.84 (m, 2H) ppm.

(3-(5-Nitro-1,3-dioxo-6-(propylamino)-1H-benzo[de]isoquinolin-2(3H)-yl)propyl)triphenylphosphonium (6)

N-Propylamine (0.16 g, 2.8 mmol) was added to a solution of 5 (1.63 g, 2.65 mmol) in MeCN (50 mL). The mixture was stirred for 5 h at room temperature under nitrogen before the solvent was evaporated under vacuum. The obtained hygroscopic residue was taken forward in synthesis without further characterisation.

(3-(5-amino-1,3-dioxo-6-(propylamino)-1H-benzo[de]isoquinolin-2(3H)-yl)propyl)triphenylphosphonium (7)

Stannous chloride dihydrate (0.51 g, 2.2 mmol, 8 equiv.) was added to a solution of the crude residue 6 in 20 mL MeOH containing 32% HCl (3 mL) under a nitrogen atmosphere. The suspension was heated to reflux for 1 h. The mixture was poured onto ice prepared from deionised water (5 g) and the solution adjusted to pH11 with 5 M NaOH. The suspension was extracted with CH2Cl2 (3 x 50 mL) and washed sequentially with water, saturated NaHCO3 solution and brine. The combined organic extracts were dried over Na2SO4 and evaporated to dryness to give 7 (213 mg) as a yellow solid which was used in the next step immediately, without further purification to prevent oxidation of the O-diamino compound in air.
	riphenyl(3-(4,6,9,11-tetraoxo-13-propyl-9,10,11,13-tetrahydro-4H-benzo[4,5]isoquinolino[7,6-g]pteridin-5(6H)-yl)propyl)phosphonium (NpFR2)

Alloxan monohydrate (108 mg, 0.68 mmol) and boric acid (49.4 mg, 0.80 mmol) were added to a stirred solution of 7 (200 mg) in glacial acetic acid (10 mL). The solution was stirred for 7 h under nitrogen, and then diluted in 100 mL water and filtered. The suspension was then filtered and the obtained solid was dried and purified by preparative TLC using DCM : MeOH (10:1) as eluent to give NpFR2 as a bright orange solid (135 mg, 0.20 mmol, 29%). 1H NMR (500 MHz, d6-DMSO): δ 8.91 (d, J = 10, 1H), 8.72 (d, J = 10, 2H), 8.12 (t, J = 10 Hz, 1H), 7.77 (m, 15H), 4.68 (br, 2H), 4.25 (t, J = 10, 2H), 3.77 (t, J = 10, 2H), 2.22 (m, 2H), 1.86 (m, 1H), 1.05 (t, J = 10, 2H). 13C NMR (125 MHz, d6-DMSO): δ 164.0, 162.9, 160.2, 156.1, 153.9, 139.9, 134.3, 133.2, 131.2, 130.8, 128.6, 124.3, 122.2, 121.2, 119.2, 118.6, 54.6, 21.0, 20.3, 11.2. HRMS: calculated 678.22647 for [M]+ C40H33N5O4P, found 678.22652
$^1$H and $^{13}$C NMR spectra of NpFR2
CELLULAR EXPERIMENTS

Cell culture

All experiments involving cultured cells used the HeLa and RAW 264.7 murine macrophage cells. Cells were maintained in exponential growth as monolayers at 37 °C in 5% CO₂ Dulbecco’s modified eagle medium (DMEM) supplemented with 2.5 mM glutamine, 10% fetal calf serum and antibiotics as per supplier’s instructions.

Cytotoxicity studies

Cytotoxicity IC₅₀ values were determined using the MTT assay, which makes use of the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product by the mitochondrial dehydrogenase of viable cells. This insoluble formazan was quantified spectrophotometrically upon dissolution in DMSO. Approximately 1 x 10⁴ cells in 100 μL DMEM were seeded into each well of flat-bottomed 96-well plates and allowed to attach overnight. NpFR2 solutions were added to triplicate wells to give final concentrations ranging from 0 – 160 μM. Following 24 h incubation, MTT (1.0 mM) was added to each well, and the plates incubated for a further 4 h. The culture medium was removed, and DMSO (150 μL) was added. The plates were shaken for 20 seconds and the absorbance measured immediately at 600 nm in a microplate reader. IC₅₀ values were determined as the probe concentration required to reduce the absorbance to 50% of that in the untreated, control wells, and represent the mean for data from at least three independent experiments.

Imaging experiments

HeLa cells were grown in glass bottom plates (MatTek Corporation) as described above. For the control imaging experiments cells were stained separately with DMSO stock solutions of NpFR2 (20 μM, 15 min), Mitotracker deep red FM (100 nM, 15 min) and Lysotracker deep red FM (100 nM, 15 min) such that the final concentration of DMSO in cell media was less than 1%. After 15 min the cells were washed thrice with phosphate-buffered saline (PBS) and maintained in DMEM (without phenol red) supplemented with 2.3 mM glutamine and 10% foetal calf serum for the duration of imaging. Confocal images were acquired using a Leica SP5 II confocal and multi-photon microscope and a UPLSAPO 100X oil-immersion objective lens. Excitation light of 488 and 633 nm was provided by the argon and HeNe lasers respectively. Cells were imaged in a temperature controlled incubator at 37 °C. Images were collected and processed using Leica Application Suite Advanced Fluorescence lite Version: 2.8.0 build 7266 viewer software. Co-localisation analyses were performed using Fiji-ImageJ-Coloc2 plugin.

Animals, cell isolation and flow cytometry

All animal studies were performed in accordance with animal ethical guidelines as approved by the Animal Ethics Committee at the University of Sydney. Adult Quackenbush Swiss male mice were killed by cervical dislocation. Bone marrow, thymus and spleen were immediately dissected. Bone marrow single cell suspensions were prepared by flushing the femora with 5 mL of PBS with a 22G needle and syringe. Thymus and spleen single cell suspensions were prepared by passing the tissues through a 40 μm mesh with the plunger of a 5 mL syringe. All single cell suspensions were then further filtered through 20 μm nylon mesh to remove clumps. Approximately 1 x 10⁶ cells were used per stain. Cells were aliquoted and treated with PBS, DMSO, NAC or H₂O₂ for 30 min at 37 °C, washed and incubated with 20 μM NpFR2 for 15 min at RT. Cells were then washed and incubated
with antibodies recognising surface proteins of live mouse haematopoietic cells for 30 min, washed again with PBS and resuspended in FACS buffer (PBS+0.5% BSA+ 1 µM propidium iodide). Cells were immediately analysed using a BD FACSCan 4-colour flow cytometer. Data was analysed using the Flowjo software package (Treestar, Ashland, OR, USA).

**Table 1:** Antibodies used in this study

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<th>Antigen</th>
<th>Clone</th>
<th>Fluorochrome</th>
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Figure S1: Absorption (red) and fluorescence (black) spectra of NpFR2. Excitation was provided at 488 nm. Spectra were acquired in HEPES buffer (100 mM, pH 7.4).
Figure S2: Fluorescence response of NpFR2 (10 µM, \(\lambda_{ex} = 488\) nm, \(\lambda_{em} = 490 - 600\) nm) to cycles of oxidation and reduction. Reduction was achieved with sodium dithionite (50 µM) followed by re-oxidation with 100 µM H\(_2\)O\(_2\). Spectra were recorded 5 min after the addition of reducing and oxidising agents. All spectra were acquired in HEPES buffer (100 mM, pH 7.4).
Figure S3: The fluorescence emission from NpFR2 (10 µM) over time. Bars represent the integrated emission intensity ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 490 - 600$ nm). NpFR2 was reduced with sodium dithionite (100 µM) and re-oxidised with hydrogen peroxide (250 µM). All spectra were acquired in HEPES buffer (100 mM, pH 7.4). Error bars represent standard deviation.
Figure S4: Oxidation of NpFR2 with various oxidising agents. Bars represent the increase in integrated emission intensity ($\lambda_{em}$ 490 - 600 nm, $\lambda_{ex} = 488$ nm) upon re-oxidation of reduced NpFR2 (10 µM in 50 µM sodium dithionite) immediately (grey) and 30 minutes after addition of 100 µM of oxidising agent. Error bars represent standard deviation.
Figure S5: The fluorescence emission of NpFR2 (10 µM) in the presence of common metal ions (100 µM). Bars represent the integrated emission intensity ($\lambda_{em} 490 - 600$ nm, $\lambda_{ex} = 488$ nm) for oxidised (black) and reduced (grey) forms. Error bars represent standard deviation.
Figure S6: The fluorescence emission from NpFR2 (10 µM) over a range of pH values. Bars represent the integrated emission intensity ($\lambda_{em} = 490 - 600$ nm, $\lambda_{ex} = 488$ nm) for oxidised (black) and reduced (grey) forms. Error bars represent standard deviation.
Figure S7: Confocal microscopy images of RAW 264.7 cells (a) untreated cells, (b) NpFR2 (20 µM, 15 min), (c) Mitotracker deep red (100 nM, 15 min), (d) Lysotracker deep red (100 nM, 15 min), (e) co-stained with NpFR2 (20 µM), and Mitotracker deep red (100 nM) for 15 min, and (f) co-stained with NpFR2 (20 µM), and Lysotracker deep red (100 nM) for 15 min, in channel 1(a1-f1) (λ<sub>ex</sub> = 488 nm, λ<sub>em</sub> = 495-600 nm), channel 2 (a2-f2) (100 nM, λ<sub>ex</sub> = 633 nm, λ<sub>em</sub> = 650-750 nm) and merged images of channel 1 and 2 (a3-f3). Intensity correlation plots of NpFR2 (x-axis) vs Mitotracker deep red (y-axis) (e4), and (d) Lysotracker deep red (y-axis)(f4).
Figure S8: Confocal microscopy images of HeLa cells (a) untreated cells, (b) NpFR2 (20 µM, 15 min), (c) Mitotracker deep red (100 nM, 15 min) and (d) co-stained with NpFR2 (20 µM), and Mitotracker deep red (100 nM) for 15 min, in channel 1 (a1-d1) ($\lambda_{ex}=488$ nm, $\lambda_{em}=495-600$ nm), channel 2 (a2-d2) ($\lambda_{ex}=633$ nm, $\lambda_{em}=650-750$ nm) and merged images of channel 1 and 2 (a3-d3). Intensity correlation plots of NpFR2 (x-axis) vs Mitotracker deep red (y-axis) (d4).