Electronic Supplementary Material (ESI) for Metallomics.

Supplementary information

Studies of the labile lead pool using a rhodamine-based fluorescent probe

Jianping Zhu,[1] Jia Hao Yeo,[1] Amy A. Bowyer,[1] Nicholas Proschogo,[1,2] Elizabeth J. New[1,2]

1. University of Sydney, School of Chemistry, Sydney, NSW, 2006, Australia
2. University of Sydney, Sydney Nano Institute, Sydney, NSW, 2006, Australia

1. Materials

All chemicals were purchased from Combi-Blocks or Merck and used without further purification. Solvents were laboratory grade. Aqueous solutions were prepared using Milli Q water. The stock solutions of metal ions were prepared from $FeCl_3$ $6H_2O$, $Fe(NH_4)_2(SO_4)_2$ $6H_2O$, $Cu(NO_3)_2$ $3H_2O$, $Zn(NO_3)_2$ $6H_2O$, $Ba(OH)_2$ $8H_2O$, $Pb(NO_3)_2$, $HgCl_2$, $Ag(NO_3)_2$, $MnCl_2$ $2H_2O$, $Co(NO_3)_2$ $6H_2O$, $CrCl_3$ $6H_2O$, $Ni(NO_3)_2$ $6H_2O$, $Cd(NO_3)_2$ $4H_2O$, KCl, $CaCl_2$, NaCl, $MgCl_2$ $6H_2O$, $AlCl_3$, CuI with Milli Q water.

2. Instrumentation

Fluorescence and UV-vis absorption spectra were recorded on Perkin Elmer Enspire Multimode Plate Reader. ^1H NMR and ^{13}C NMR spectra were collected on Bruker DRX 300 spectrometer. Low-resolution ESI mass spectrometry was performed on a Bruker Amazon SL mass spectrometer. High-resolution mass spectrometry was collected on a Thermo LTQ Orbitrap XL mass spectrometer. Inductively coupled plasma mass spectrometry (ICP-MS) was recorded on Perkin Elmer Nexion 350X Inductively Coupled Plasma Mass Spectrometer. Single Cell ICP-MS was collected on Perkin Elmer Nexion 2000B Inductively Coupled Plasma Mass Spectrometer.

3. Cellular experiments

Cell culture

DLD-1 cells (passage number below 20) were cultured in a humidified 5% CO_2 atmosphere at 37 °C in advanced DMEM (Dulbecco's modified eagle medium) supplemented with 2% fetal bovine serum (v/v) and 2 mM glutamine. DLD-1 cells were passaged every 2-3 days at a density of 1.0 x 10^6 cells.

K562 cells (passage number below 20) were cultured in a humidified 5% CO_2 atmosphere at 37 °C in advanced RPMI (Roswell Park Memorial Institute) medium supplemented with 5% fetal bovine serum (v/v) and 2 mM glutamine. K562 cells were passaged every 2-3 days at a density of 0.5 x 10^6 cells.

Confocal imaging experiments

Confocal images were collected on Olympus FluoView FV3000 Confocal microscope with Olympus UPLanSApo 60x water-immersion objective lens (NA = 1.20). DLD-1 cells were cultured as described above. Cells were suspended in advanced DMEM. 1.0 x 10^4 cells were seeded onto 35 mm glass-bottom dishes (MatTek Corporation), then incubated in a humidified 5% CO_2 atmosphere at 37 °C for 24 h to make sure the adherence of cells. Cells were treated with 1 mL of advanced DMEM containing $Pb(NO_3)_2$ (50 μM) for 4 h, washed with PBS for 3 times, then incubated with 1 mL of advanced DMEM containing **RPb1** (50 μM) for 30 min, washed with PBS for 3 times. 1 mL of FluoroBrite DMEM supplemented with 2% fetal bovine serum (v/v) and 2 mM glutamine was added into cells before imaging.

For colocalisation experiments, cells were pretreated with 1 mL of advanced DMEM containing $Pb(NO_3)_2$ (50 μM) for 4 h, washed with PBS for 3 times, then incubated with 1 mL of advanced DMEM containing RPb1 (50 μM) and LysoTracker Blue (100 nM) for 30 min, washed with PBS for 3 times. 1 mL of FluoroBrite DMEM supplemented with 2% fetal bovine serum (v/v) and 2 mM glutamine was added into cells before imaging.

Flow cytometry experiments

Approximately 2.0 x 10^6 K562 cells were treated with haemin (30 μM) for different time, washed with PBS for 3 times, followed by treatment of $Pb(NO_3)_2$ (50 μM) for 4 h in a humidified 5% CO_2 atmosphere at 37 °C. Then cells were incubated with **RPb1** (50 μM) for 30 min, washed with PBS for 3 times and assessed on Gallios Flow Cytometer (5+3+2 configuration). A total number of 1 x 10^6 events were collected and analysed. Data collected from the Gallios flow cytometer were analysed on FlowJo version 10.6 (TreeStar)

Single cell ICP-MS experiments

Approximately 1.0 x 10^6 K562 cells were treated with haemin (30 µM) at different time-points and incubated in the cell culture incubator (humidified 5% CO_2 atmosphere at 37 °C). Cells were washed with PBS for 3 times, followed by treatment with $Pb(NO_3)_2$ (50 µM) for 4 h in the cell culture incubator. Cells were again washed with PBS for 3 times and resuspended to a final concentration of 100,000 cells/mL. Lead content in cells were assessed using Perkin Elmer NexION 2000B Inductively Coupled Plasma Mass Spectrometer (coupled to an ESI-SC-µ DX autosampler), with a Sample Flow Rate of 0.01 mL/min with PBS as rinse solvent. Measurements were performed with a 50 µs Dwell Time over a Scan Time of 60 sec. Data acquired were exported from the SC-ICP-MS and were analysed on GraphPad Prism.

4. Synthesis

Rhodamine B hydrazide (1) was synthesised according to literature procedures.[1] A solution of rhodamine B (2.3 mmol, 1.10 g) and hydrazine monohydrate (1.12 mL) in methanol (30 mL) was heated under reflux overnight. Then reaction solution was poured into distilled water (50 mL) and extracted with ethyl acetate (3 x 50 mL). The extract was dried with anhydrous sodium sulfate, filtered and evaporated. Crude residue was purified by column chromatography on silica gel (MeOH : CH_2Cl_2, 1 : 30) to give compound **1** as pink solid (516.8 mg, 49.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95-7.93 (m, 1H), 7.46-7.44 (m, 2H), 7.11-7.09 (m, 1H), 6.47-6.43 (m, 4H), 6.30 (d, J = 9 Hz, 2H), 3.62 (s, 2H), 3.34 (q, J = 6 Hz, 8H), 1.17 (t, J = 6 Hz, 12H).LR-ESI-MS: $C_{28}H_{32}N_4O_2$, $[M+Na]^+$, found 479.27, calculated 479.58.

Methyl 6-(hydroxymethyl)picolinate (2) was synthesised following literature procedures.[2] Sodium tetrahydridoborate (10.31 mmol, 390 mg) was slowly added at 0 °C to a solution of dimethyl pyridine-2,6-dicarboxylate (10.25 mmol, 2.0 g) in a dry 7 : 3 mixture of MeOH / CH_2Cl_2 (100 mL). The reaction mixture was stirred for 3 h at rt and then neutralised with an aqueous saturated NH_4Cl solution (50 mL). After extraction with CH_2Cl_2 (3 x 50 mL), the combined organic extract was dried with anhydrous sodium sulfate and solvent was removed under reduced pressure. The resulting crude residue was purified by column chromatography (hexane : ethyl acetate, 1 : 1) giving compound **2** as white solid (1.03 g, 60.05%). ^{1}H NMR (300 MHz, MeOD): δ 8.04-7.95 (m, 2H), 7.79-7.75 (m, 1H), 4.75 (s, 2H), 3.97 (s, 3H). LR-ESI-MS: $C_8H_9NO_3$, $[M+Na]^+$, found 190.02, calculated 190.05; $[2M+Na]^+$, found 357.05, calculated 357.11.

Methyl 6-(bromomethyl)picolinate (3) was synthesised according to literature.[2] Phosphorus tribromide (11.1 mmol, 1.04 mL) was added at 0 °C to a solution of compound 2 (10.3 mmol, 1.72 g) in anhydrous $CHCl_3$ (150 mL). The reaction mixture was stirred for 4 h at rt, then neutralised at 0 °C with aqueous saturated K_2CO_3 solution (100 mL). After extraction with CH_2Cl_2 (2 x 50 mL), the combined organic extract was dried with Na_2SO_4 and the solvent was removed under reduced pressure, leading to pure compound **3** as white solid (2.11 g, 89.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.08-8.05 (m, 1H), 7.90-7.83 (m, 1H), 7.71-7.67 (m, 1H), 4.65 (s, 2H), 4.02 (s, 3H). ESI-MS: $C_8H_8BrNO_2$, $[M+Na]^+$, found 251.98, calculated 251.96; $[2M+Na]^+$, found 482.89, calculated 482.93.

RPb1. A solution of rhodamine B hydrazide (1 mmol, 456.59 mg), methyl 6-(bromomethyl)picolinate (2 mmol, 460.12 mg) and potassium carbonate (2 mmol, 276.40 mg) in dry CH_3CN was heated under reflux overnight. Upon completion, solvent was removed. The residue was extracted with CH_2Cl_2 and washed with water. Organic layer was dried with Na_2SO_4 and concentrated, then purified by column chromatography (MeOH : CH_2Cl_2, 1 : 60). **RPb1** was obtained as an off white solid (218.92 mg, 29%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.97-7.92 (m, 1H), 7.68 (d, J = 6 Hz, 2H), 7.49-7.44 (m, 2H), 7.35 (t, J = 7.5 Hz, 2H), 7.07-7.01 (m, 1H), 6.83 (d, J = 6 Hz, 2H), 6.33 (s, 1H), 6.30 (s, 1H), 6.28 (d, J = 3 Hz, 2H), 6.02 (d, J = 3 Hz, 1H), 5.99 (d, J = 3 Hz, 1H), 4.72 (d, J = 15 Hz, 2H), 4.30 (d, J = 15 Hz, 2H), 3.88 (s, 6H), 3.26 (q, J = 8 Hz, 8H), 1.09 (t, J = 7.5 Hz, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): 166.34, 165.85, 159.27, 154.07, 151.20, 148.50, 146.35, 136.29, 132.77, 130.99, 129.24, 128.22, 126.55, 124.18, 122.86, 122.69, 107.81, 105.92, 97.60, 65.65, 63.78, 52.59, 44.28, 12.58. HR-ESI-MS: $C_{44}H_{46}N_6O_6$, $[M+Na]^+$, found 777.3371, calculated 777.3371.

5. Supporting figures

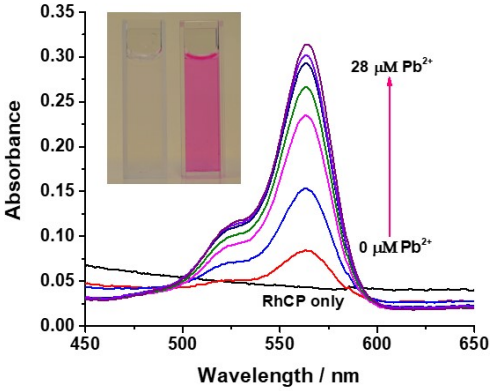


Fig. S1 UV-vis absorbance spectra of **RPb1** (20 μM) in the presence of various amounts of Pb^{2+} (0-28 μM) in HEPES buffer (20 mM, pH 7.4, containing 2% DMSO as a cosolvent, v/v). The inset shows color of **RPb1** (20 μM) before (left) and after (right) addition of Pb^{2+} (20 μM).

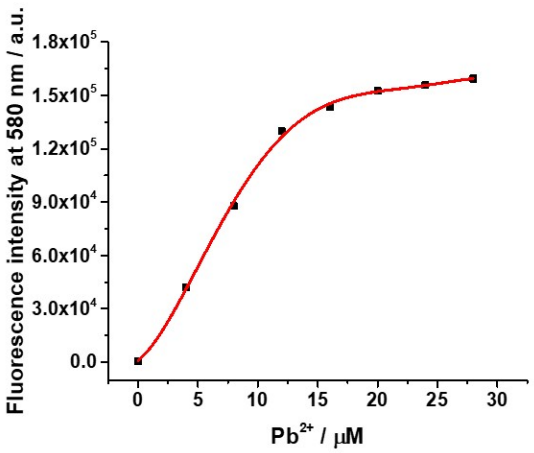


Fig. S2 Fluorescence intensity of **RPb1** (20 μM) at 580 nm with increasing concentrations of Pb^{2+} in HEPES buffer (20 mM, pH 7.4, containing 2% DMSO as a cosolvent, v/v).

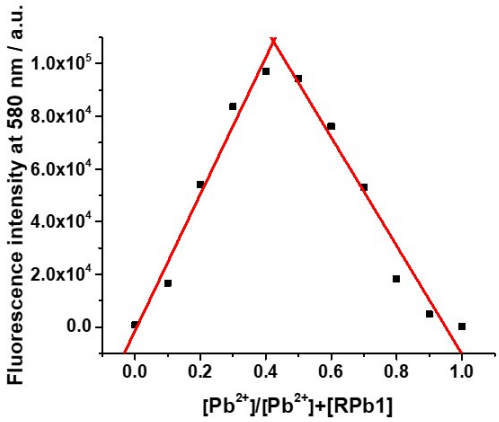


Fig. S3 Job's plot of **RPb1** with Pb^{2+} in HEPES buffer (20 mM, pH 7.4).

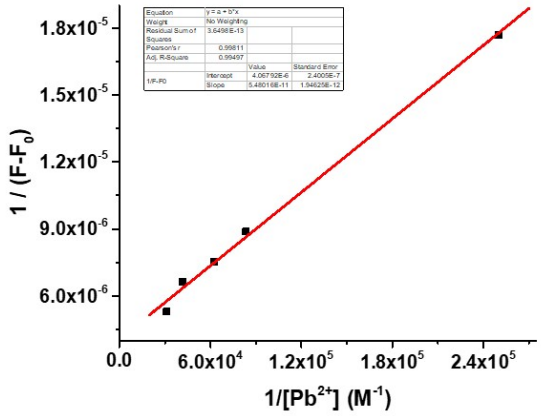


Fig. S4 Benesi-Hildebrand plot of **RPb1** (20 μM) with different concentrations of Pb^{2+} in HEPES buffer (20 mM, pH 7.4, containing 2% DMSO as a cosolvent, v/v). $y = 4.06792 \times 10^{-6} + 5.48016 \times 10^{-11}x$

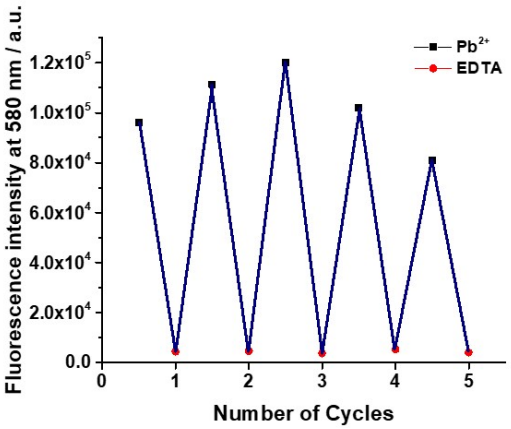


Fig. S5 Fluorescence intensity of **RPb1** (20 μM) at 580 nm upon alternative addition of Pb^{2+} (20 μM) and EDTA (20 μM) in HEPES buffer (20 mM, pH 7.4). Number of cycles refers to alternating Pb^{2+}/EDTA cycles.

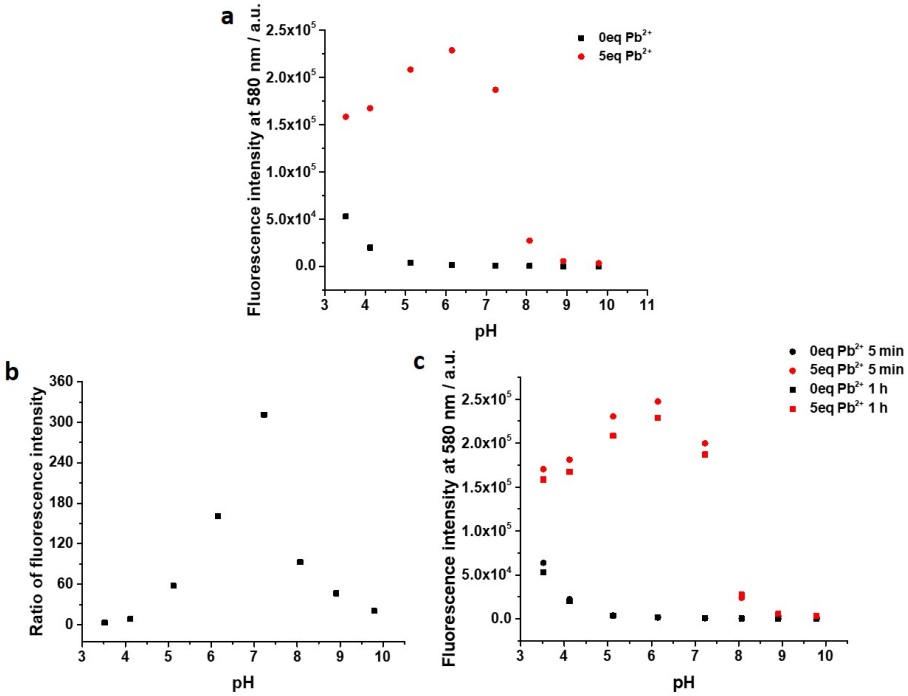


Fig. S6 (a) Fluorescence intensity of **RPb1** (20 μM) at 580 nm in the absence and presence of Pb^{2+} at different pH values in HEPES buffer (20 mM, containing 2% DMSO as a cosolvent, v/v). (b) Ratio of fluorescence intensity of **RPb1** at 580 nm in the presence of Pb^{2+} to that in the absence of Pb^{2+}. (c) Fluorescence stabilities of **RPb1** (20 μM) at 580 nm at different pH values in HEPES buffers after treatment of Pb^{2+} for 5 min and 1 h.

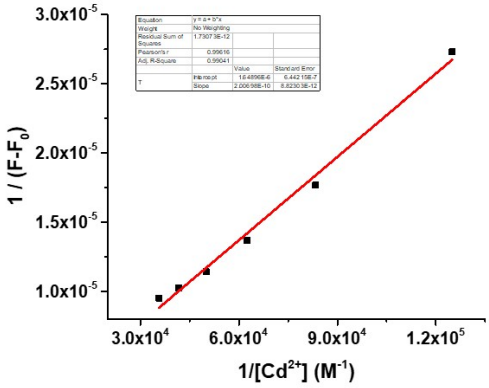


Fig. S7 Benesi-Hildebrand plot of **RPb1** (20 μM) with different concentrations of Cd^{2+} in HEPES buffer (20 mM, pH 7.4, containing 2% DMSO as a cosolvent, v/v). $y = 1.64896 \times 10^{-6} + 2.00698 \times 10^{-10}x$

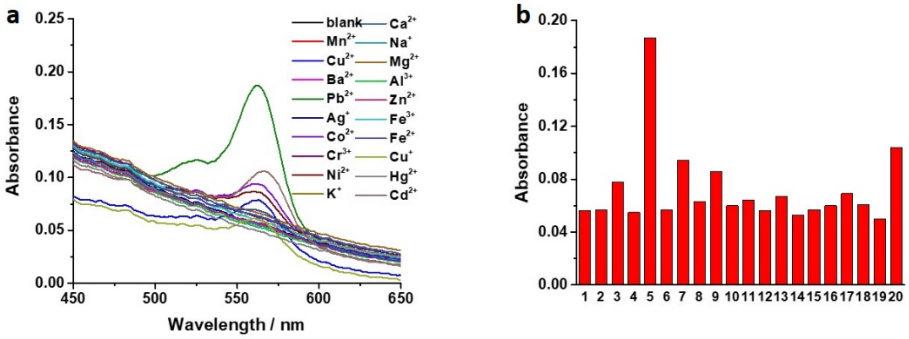


Fig. S8 (a) UV-vis absorbance spectra of **RPb1** (20 μM) in the presence of various metals (100 equivalent for K^+, Ca^{2+}, Na^+ and Mg^{2+}, one equivalent for others) in HEPES buffer (20 mM, pH 7.4, containing 2% DMSO as a cosolvent, v/v). (b) Absorbance of **RPb1** at 562 nm after addition of various metals (1. blank; 2. Mn^{2+}; 3. Cu^{2+}; 4. Ba^{2+}; 5. Pb^{2+}; 6. Ag^+; 7. Co^{2+}; 8. Cr^{3+}; 9. Ni^{2+}; 10. K^+; 11. Ca^{2+}; 12. Na^+; 13. Mg^{2+}; 14. Al^{3+}; 15. Zn^{2+}; 16. Fe^{2+}; 17 Fe^{3+}; 18. Cu^+; 19. Hg^{2+}; 20. Cd^{2+}).

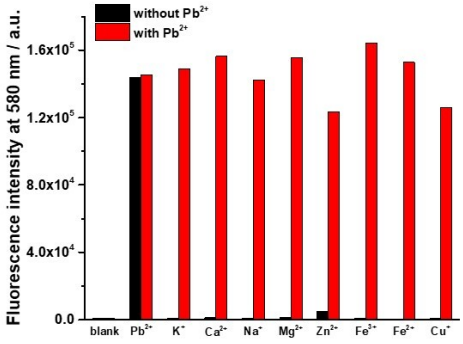


Fig. S9 Fluorescence intensity of **RPb1** (20 μM) at 580 nm (λ_{ex} = 526 nm) after addition of various metals (100 equivalents for K^+, Ca^{2+}, Na^+ and Mg^{2+}, one equivalent for others) followed by Pb^{2+} (one equivalent).

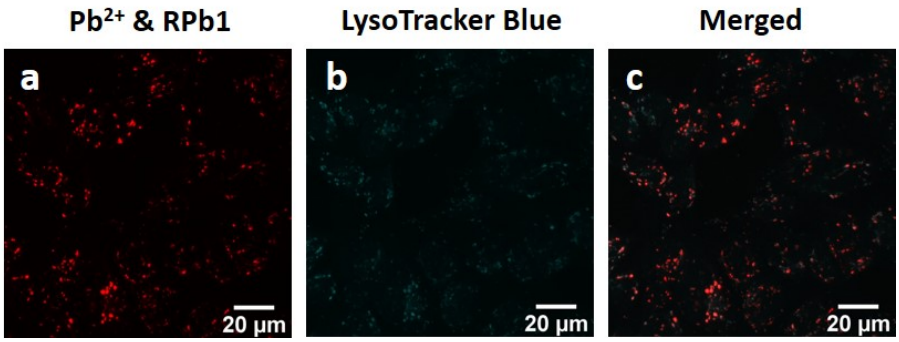


Fig. S10 Colocalisation images of DLD-1 cells pretreated with Pb^{2+} (50 μM) for 4 h, then coincubated with **RPb1** (50 μM) and LysoTracker Blue (100 nM) for 30 min. (a) Fluorescence channel of **RPb1** (566 - 666 nm) excited with 561 nm laser. (b) Fluorescence channel of LysoTracker Blue (410 - 440 nm) excited with 405 nm laser. (c) Merged image of **RPb1** channel with LysoTracker Blue channel.

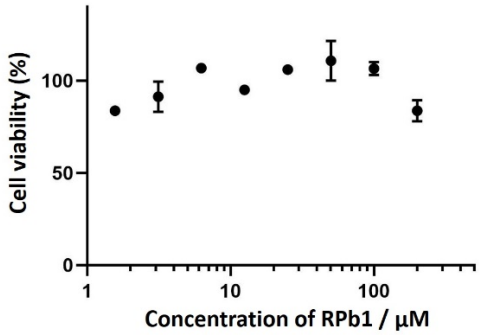


Fig. S11 Cytotoxicity test of **RPb1** in DLD-1 cells over 4 h determined by Alamar Blue staining.

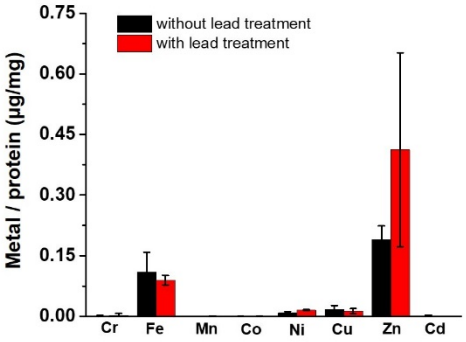


Fig. S12 Concentrations of transition metals in DLD-1 cells treated with or without Pb^{2+} at 37 °C measured by ICP-MS.

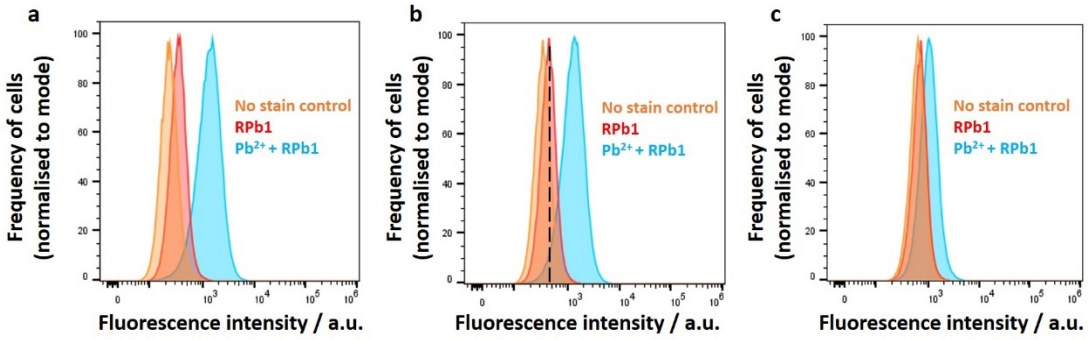


Fig. S13 Representative flow cytometry plots of K562 cells with different incubation conditions. K562 Cells were pretreated with haemin (30 µM) for 0 h (a), 1 h (b) and 5 h (c), then incubated with or without Pb^{2+} (50 µM) for 4 h, followed by treatment with **RPb1** (50 µM) for 30 min.

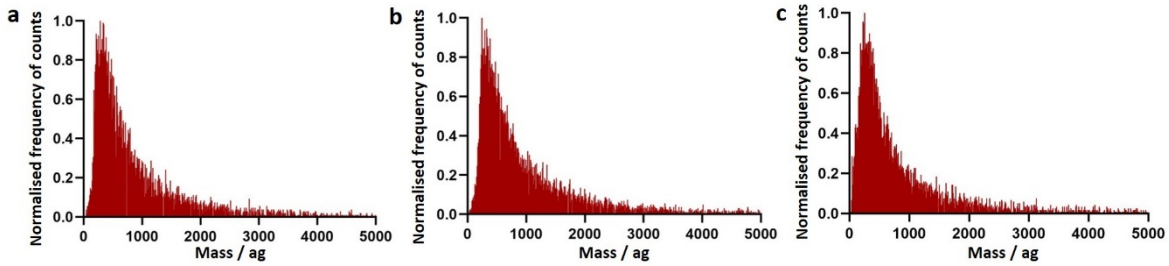


Fig. S14 Representative histograms of SC-ICP-MS for cells with different incubation conditions. (a) K562 cells were treated with Pb^{2+} (50 μM) for 4 h. (b) K562 cells were preincubated with haemin (30 μM) for 1 h, then treated with Pb^{2+} (50 μM) for 4 h. (c) K562 cells were preincubated with haemin (30 μM) for 5 h, then treated with Pb^{2+} (50 μM) for 4 h.

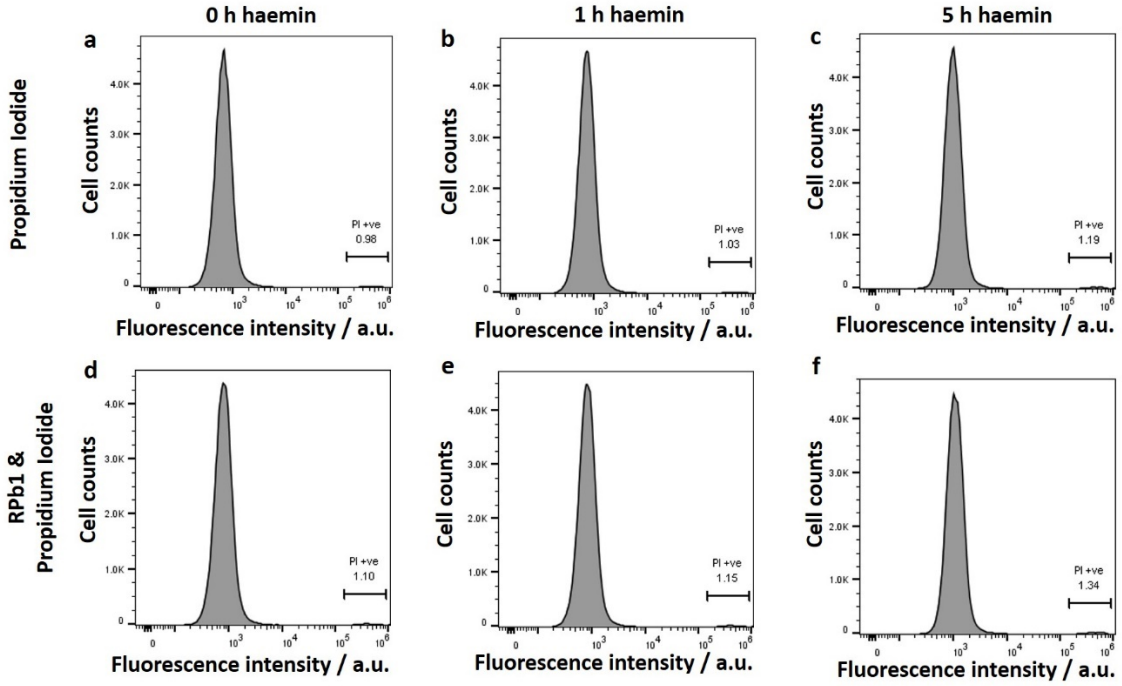
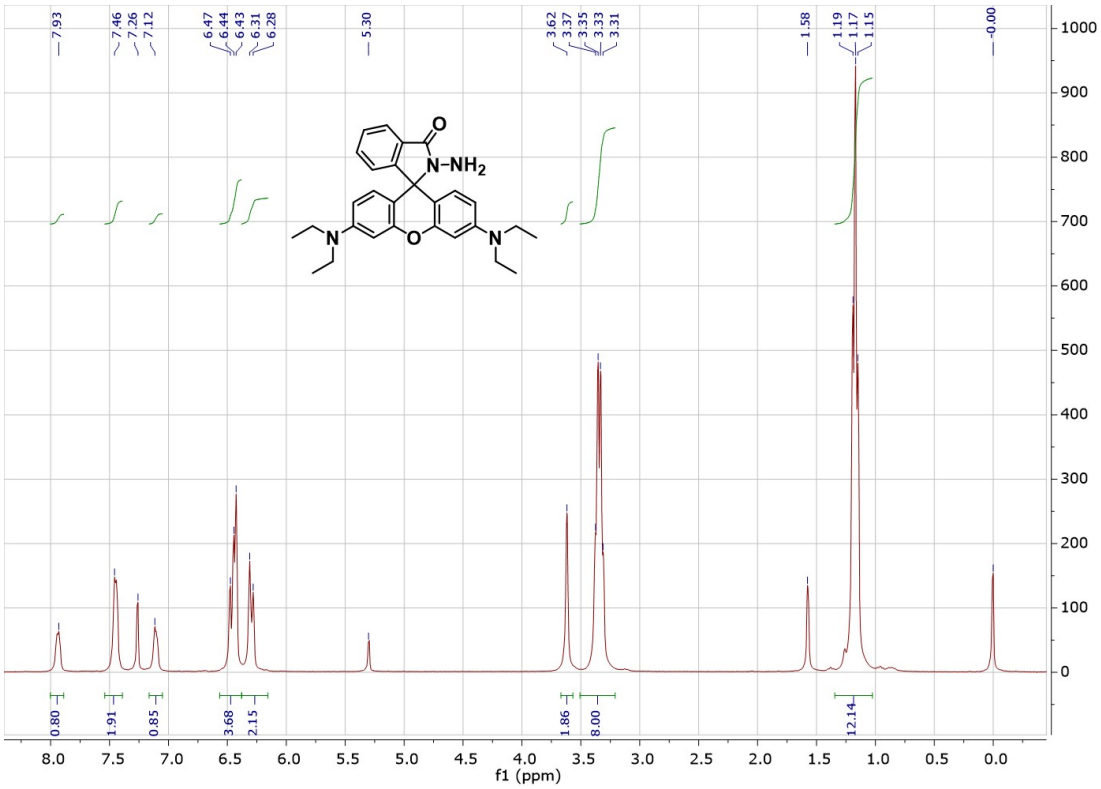
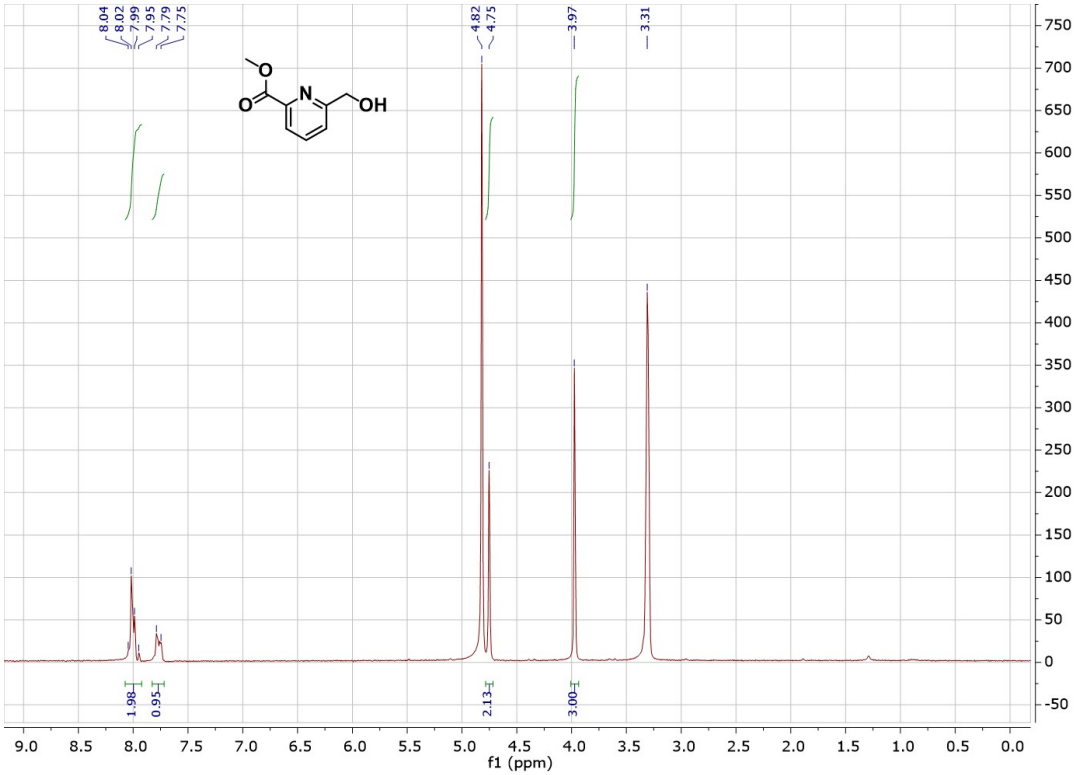


Fig. S15 Representative ungated flow cytometric histograms for cells with different incubation conditions. All cells were treated with Pb^{2+} (50 μM) for 4h, followed by treatment with haemin (30 μM) for 0 h (a, d), 1 h (b, e) and 4 h (c, f). Then cells were incubated with **RPb1** (50 μM) for 30 min (d, e, f). All cells were stained with propidium iodide (1 μg/mL, PI) prior to flow cytometry (a, b, c, d, e, f).

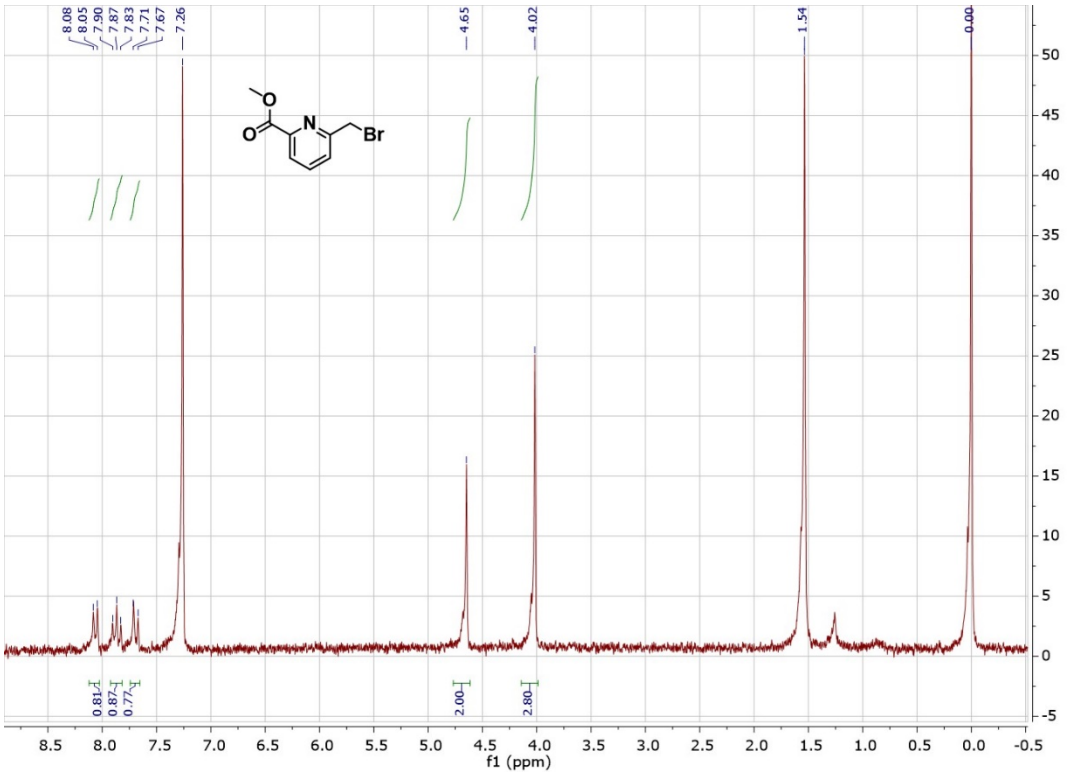
6. ^{1}H NMR and ^{13}C NMR of compounds



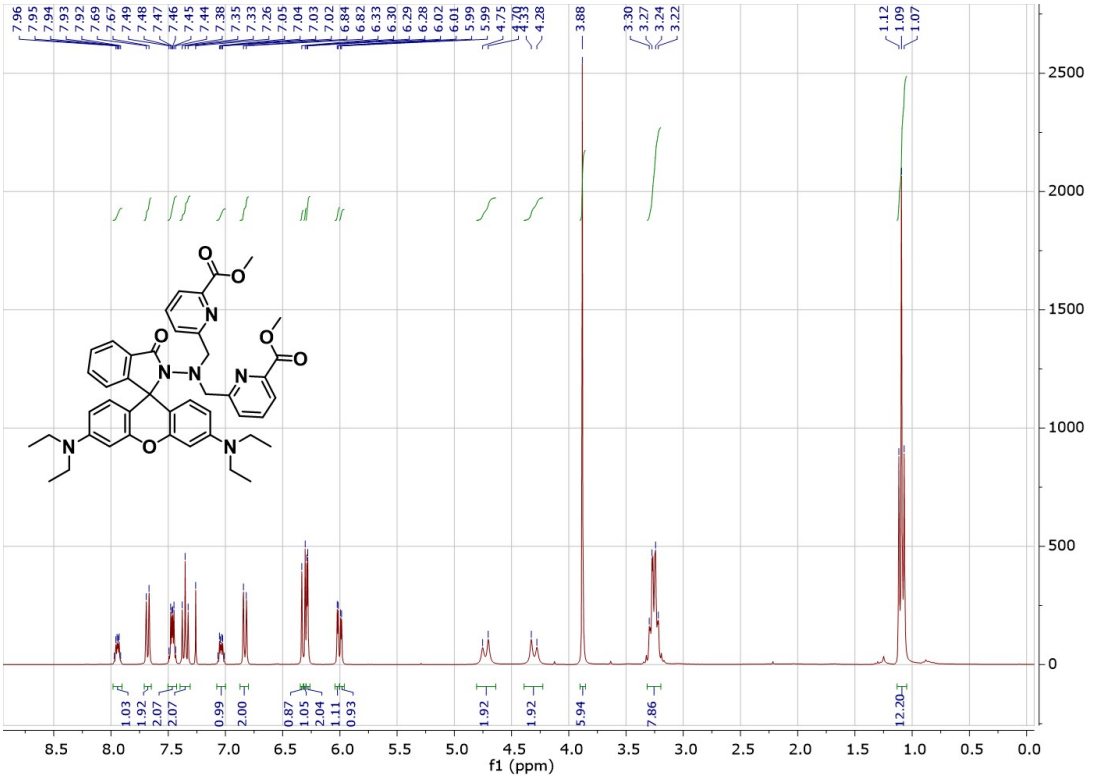
^{1}H NMR spectrum of compound **1** in $CDCl_3$.



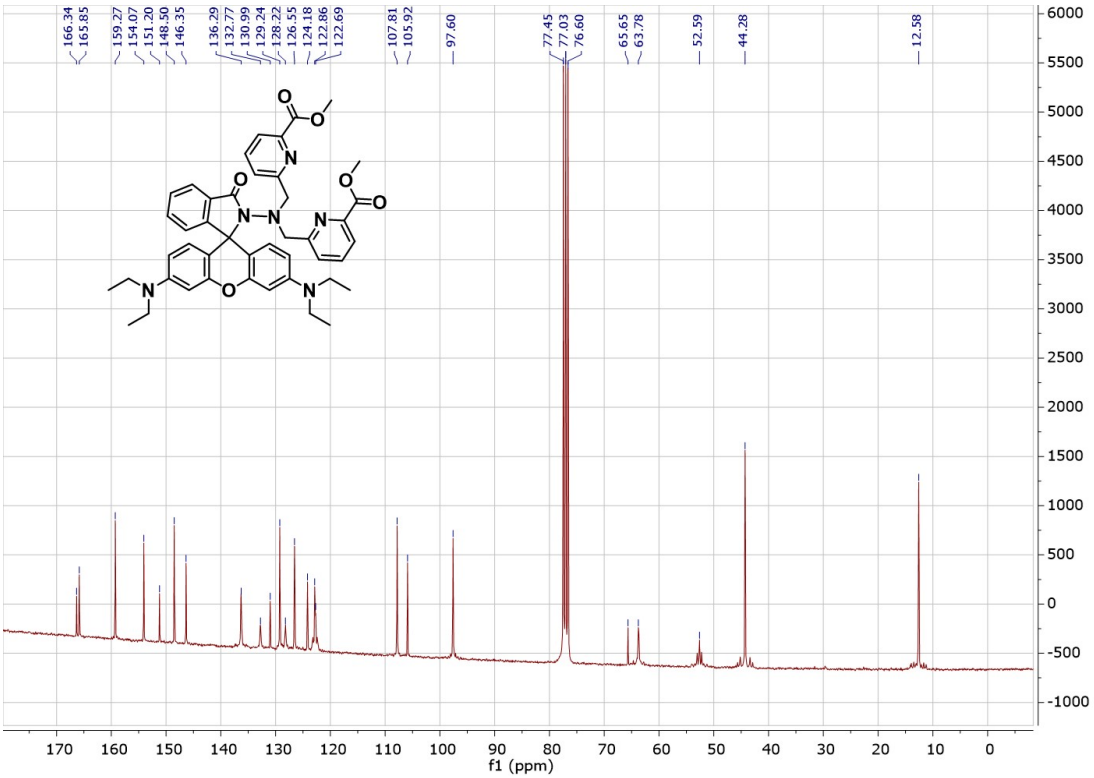
^{1}H NMR spectrum of compound **2** in MeOD.



^{1}H NMR spectrum of compound **3** in $CDCl_3$.



^{1}H NMR spectrum of **RPb1** in $CDCl_3$.



^{13}C NMR spectrum of **RPb1** in $CDCl_3$.

References

1 A. Raman, G. Augustine, N. Ayyadurai and S. Easwaramoorthi, *J. Mater. Chem. C*, 2018, **6**, 10497-10501.

2 X. Zeng, D. Coquière, A. Alenda, E. Garrier, T. Prangé, Y. Li, O. Reinaud and I. Jabin, *Chem. Eur. J.*, 2006, **12**, 6393-6402.