Supplementary information

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

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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$_3$ $\cdot$ 6H$_2$O, Fe(NH$_4$)$_2$(SO$_4$)$_2$, Cu(NO$_3$)$_2$, Zn(NO$_3$)$_2$, 6H$_2$O, Ba(OH)$_2$, 8H$_2$O, Pb(NO$_3$)$_2$, HgCl$_2$, Ag(NO$_3$)$_2$, MnCl$_2$, 2H$_2$O, Co(NO$_3$)$_2$, 6H$_2$O, CrCl$_3$, 6H$_2$O, Ni(NO$_3$)$_2$, Cd(NO$_3$)$_2$, 4H$_2$O, KCl, CaCl$_2$, NaCl, MgCl$_2$, 6H$_2$O, AlCl$_3$, KI, with Milli Q water.

2. **Instrumentation**

Fluorescence and UV-vis absorption spectra were recorded on Perkin Elmer Enspire Multimode Plate Reader. $^1$H 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$^6$ 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 $1.0 \times 10^6$ 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$_2$Cl$_2$, 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$_4$O$_2$, [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$_2$Cl$_2$ (100 mL). The reaction mixture was stirred for 3 h at rt and then neutralised with an aqueous saturated NH$_4$Cl solution (50 mL). After extraction with CH$_2$Cl$_2$ (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$_8$H$_9$NO$_3$, [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$_2$CO$_3$ solution (100 mL). After extraction with CH$_2$Cl$_2$ (2 x 50 mL), the combined organic extract was dried with Na$_2$SO$_4$ 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$_8$H$_8$BrNO$_2$, [M+Na]$^+$, found 251.98, calculated 251.96; [2M+Na]$^+$, found 482.93, calculated 482.93.

RPb1. A solution of rhodamine B hydrazide (1 mmol, 456.59 mg) and methyl 6-(bromomethyl)picolinate (2 mmol, 460.12 mg) and potassium carbonate (2 mmol, 276.40 mg) in dry CH$_3$CN was heated under reflux overnight. Upon completion, solvent was removed. The residue was extracted with CH$_2$Cl$_2$ and washed with water. Organic layer was dried with Na$_2$SO$_4$ and concentrated, then purified by column chromatography (MeOH : CH$_2$Cl$_2$, 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$_6$O$_6$, [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 Pb²⁺ (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 Pb²⁺ (20 µM).

**Fig. S2** Fluorescence intensity of RPb1 (20 µM) at 580 nm with increasing concentrations of Pb²⁺ in HEPES buffer (20 mM, pH 7.4, containing 2% DMSO as a cosolvent, v/v).

**Fig. S3** Job’s plot of RPb1 with Pb²⁺ in HEPES buffer (20 mM, pH 7.4).
**Fig. S4** Benesi-Hildebrand plot of RPb1 (20 µM) with different concentrations of Pb²⁺ 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 Pb²⁺ (20 µM) and EDTA (20 µM) in HEPES buffer (20 mM, pH 7.4). Number of cycles refers to alternating Pb²⁺/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. S9 Fluorescence intensity of RPb1 (20 µM) at 580 nm (λex = 526 nm) after addition of various metals (100 equivalents for K⁺, Ca²⁺, Na⁺ and Mg²⁺, one equivalent for others) followed by Pb²⁺ (one equivalent).

Fig. S10 Colocalisation images of DLD-1 cells pretreated with Pb²⁺ (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 R Pb1 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\textsuperscript{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\textsuperscript{2+} (50 µM) for 4 h, followed by treatment with R Pb1 (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 CDCl₃.

\(^1\)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 CDCl₃.

References