Supporting Information (ESI) for

“Dual Selective Iron Chelating Probes with a Potential to Monitor Mitochondrial Labile Iron Pools”

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Materials

Ultra high purity water obtained from a Milli-Q system was used throughout this study for both the chemistry and biology work. PyOxP was synthesized by following a recently reported method1. NovaPEG Rink Amide resin (substitution = 0.49 mmol/g) was supplied from Merck Chemicals Ltd (Nottingham, UK). Acetone, atomic absorption Fe (1000 g/l), diethyl ether, dichloromethane (DCM), methanol, HPLC grade acetonitrile (ACN) were from Fisher Scientific UK Ltd (Loughborough, UK). Anhydrous dimethylformamide (DMF), Oxyma, hydrazine hydrate, nitrilo-triacetic acid trisodium salt (NTA), N,N’-disopropylcarbodiimide (DIC), phenol, piperidine, thioanisole (TA), trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) were supplied from Sigma-Aldrich (Gillingham, UK). Fmoc-protected amino acids and all other peptide synthesis reagents were from Bachem (Merseyside, UK), except Boc-Glu(OFm)-OH which was purchased from p3bioSystems (Louisville, USA). All reagents were used as received except DMF which was kept under a constant flow of nitrogen.

For cell cultivation, all reagents used were cell culture-grade and purchased from Life Technologies (Paisley, Scotland), except Gold foetal calf serum (FCS), which was from PAA Laboratories GmbH (Colbe, Germany) and PBS from Oxoid Ltd (Basingstoke, UK). The organelle-specific marker MitoTracker® Deep Red FM was purchased from Life Technologies (Paisley, Scotland).

Analytical

The analytical RP-HPLC was carried out on a HP1050 HPLC system equipped with an autosampler, a quaternary pump and a Diode-Array Detector. A Zorbax SB C-18 2.1mm x 10cm (particle size 5 micron) column was employed. The flow rate was 0.2 mL/min and the eluents were monitored at wavelengths between 210-280 nm. A linear gradient of mobile phase B (acetonitrile containing 0.1% TFA) over mobile phase A (0.1% TFA in water) from 0-90% B in 20 minutes was performed. Data were collected and analyzed using ChemStation software.

The semi-prep HPLC purification was performed using a X-terra Prep MS C18 Column (5 micron, 10 x 100 mm) operating a flow rate of 7 mL/min. Isolated fractions were reanalyzed via analytical RP-HPLC as above and identical fractions were pooled and lyophilized.

Low resolution ESI-MS analyses were performed using a Waters Micromass ZQ mass spectrometer (Manchester, UK). HRES-MS analysis was carried out by the Mass Spectrometry Facility, King’s College London, on an Exactive Plus Orbitrap Mass Spectrometer.

1H NMR and 13C NMR spectra were recorded using a Bruker Avance 400 (400 MHz) NMR spectrometer. Chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). J values are in hertz (Hz), and splitting patterns are designated as follows: s (singlet), bs (broad singlet), d (doublet), t (triplet), and m (multiplet).
UV-vis and fluorescence measurements were performed on a Perkin Elmer spectrophotometer and a Perkin Elmer spectrofluorometer, respectively. Probe solutions were prepared at the appropriate concentrations in 0.1 M MOPS buffer, pH 7.4.

**Synthesis of iron chelators**

![Scheme S1: Synthesis of compounds 1-2.](image)

Intermediates A-E (Scheme S1) were prepared according to a previously reported procedure \(^2\). Compounds 9 and 10 were synthesized in an analogous manner to that recently described\(^3\). Addition/cyclization reactions between E or C with 3,4-diaminobenzoic acid to afford compounds 2 or 1, respectively, was performed by modification of a recently reported method\(^3\) as follows:

9-(benzyloxy)-6,8-dioxo-6,8-dihydro-5H-pyrido[1,2-a]quinoxaline-2-carboxylic acid (1) and 9-methoxy-6,8-dioxo-6,8-dihydro-5H-pyrido[1,2-a]quinoxaline-2-carboxylic acid (2): compound C (1 g, 4 mmol) and 3,4-diaminobenzoic acid (1.3 g, 8.5 mmol) were suspended in a 1:1 v/v water/ethanol solution (100 mL) and the mixture was refluxed overnight. Upon cooling, the formed solid was isolated by centrifugation, dissolved in hot ethanol, and cooled to afford a dark solid. This solid was centrifuged, washed with cold ethanol and dried to afford the product as a dark solid. The analytical RP-HPLC of this product (Fig. S1) showed one predominant peak with a close-eluting side-peak, suggesting the presence of a main stereoisomer with a small amount of a second stereoisomer. This was further confirmed by NMR, where about 10% (calculated from the integrals of the benzyl protons) of the second isomer was observed. The proposed main stereoisomer is the one presented in Fig. 2 and Scheme S1 (compound 1) as we predicted the amine in position 3 (in 3,4 diaminobenzoic acid) to be a stronger nucleophile and as judged by NMR assignment. Therefore, addition to the maltol by 3,4 diaminobenzoic acid is favoured in position 3, generating one preferred isomer. However, the reaction conditions, namely reaction time and temperature, play a critical role, as the previously reported method\(^3\) produced compound 1 as isomeric mix in approximately 2:1 ratio. Found m/z 363.0904 (calculated 363.0981) [M+H]\(^+\).

An analogous procedure using compound E afforded 2 as brown solid. Found m/z 287.0661 (calculated 287.0668) [M+H]\(^+\).
Sodium salts of compounds 1 and 2:
Compound 1 was dissolved in 3% NaOH solution (to a total one equivalent base with respect to carboxylic acid content), diluted with methanol and a 10-fold excess of cold diethyl ether was added to afford a crystalline solid. The solid was isolated via centrifugation and washed (x2) with diethyl ether, and finally dried under high-vacuum to afford the sodium salt (65% overall yield from 1). The analytical RP-HPLC of this product showed one single peak (Fig. S1), suggesting that one single isomer preferentially crystallized as the sodium salt. This was further confirmed by NMR analysis, whereby by comparing the integrals of the benzyl protons a >95% stereisomeric pure species was identified. $^1$H NMR (D$_2$O, 400 MHz): $\delta$ 4.74 (s, 2H), 6.72 (d, $J$ = 8.3 Hz, 1H), 6.88 (s, 1H), 7.17-7.45 (m, 6H), 7.64 (s, 1H) and 7.79 (s, 1H) ppm. $^{13}$C NMR (D$_2$O, 100.62 MHz): $\delta$ 70.81, 112.49, 114.07, 114.83, 119.81, 121.07, 127.63, 128.24, 128.32, 128.56, 131.63, 132.97, 134.89, 150.43, 156.77, 170.84 and 172.20 ppm. An analogous procedure using compound 2 afforded its sodium salt as brown solid.

Fig. S1 HPLC traces of compounds 1 and 2.

Peptide Synthesis
The peptides were manually synthesized employing an orthogonal Fmoc-SPPS method using empty fritted polypropylene tubes. The diamino propionic acid (DAP) or lysine residues that were designed for site-selective conjugation to an iron chelator were protected at the side-chains with a [1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] (Dde) group. D-arginine was incorporated in position 3 of all the peptides to increase peptide stability towards hydrolase-enzymes and it bore a (2,2,4,6,7-
pentamethyldihydrobenzofuran-5-sulfonyl) (Pbf) protecting group at the side chain. Lysines not taking part into iron chelator-peptide couplings were orthogonally protected with a Boc group. Boc-Phe-OH was incorporated in the peptide sequence in all the N-terminal Phe peptides. For the preparation of compounds 11-12, a Boc-Glu(OFm)-OH residue was incorporated at the C-terminal site for the site-selective conjugation of amine-containing iron chelators 9 or 10, respectively. The amide linker-resin was swollen in anhydrous DMF for thirty minutes before the first coupling reaction. The acylation steps were performed using four equivalents of the Fmoc amino acids, which were pre-activated with Oxyma and DIC in DMF in a molar ratio of 1:1:1 of amino acid, Oxyma and DIC, respectively. Coupling reactions were conducted for two-three hours and monitored by the picrylsulphonic acid test. The coupling of Fmoc-D-Arg(Pbf)-OH was repeated to ensure complete acylation. Fmoc deprotection of the coupled amino acids was achieved by treatment with 20% (v/v) piperidine in DMF for ten minutes. Successful deprotection was confirmed by picrylsulfonic acid test. Excess of reagents and impurities were removed by extensive washing with DMF, methanol and DCM. The Dde-protecting group was selectively removed by treating the resin with a solution of 2% v/v hydrazine hydrate in DMF (6 x 10 minutes). Following completion of the synthesis, the resin was washed with DMF and methanol and dried in vacuo for three hours at room temperature (RT). The cleavage of the peptides from the solid support, along with the simultaneous removal of the acid-labile side-chain protecting groups, was achieved by treating the peptide-resin with a solution of TFA containing 5% v/v water, 5% w/v phenol, 5% v/v TA and 2% TIPS for three hours at RT with gentle shaking. Thereafter, the resin was filtered off, washed with TFA, and concentrated under a stream of nitrogen at 40 °C. Ice-cold diethyl ether was added to precipitate the peptides, and the organic solvent was removed by centrifugation. The resulting peptides were washed several times with diethyl ether to remove any residual scavenger traces, dissolved in 50% ACN in water containing 0.1% TFA and freeze-dried for twenty-four hours. Finally, the peptides were purified by RP-HPLC using water/ACN gradients containing 0.1% TFA using a X-terra Prep-MS C18 column. The purity and correct mass of the final peptides were confirmed by analytical RP-HPLC and HRMS, respectively. For the site-specific conjugation of the protected iron chelator 1 into the peptide backbone, the protected chelator (four-fold excess) was pre-activated for five minutes with an equimolar amount of PyOxP and two-fold excess DIPEA in DMF and then added to the corresponding peptide-resin bearing a free-amino group of either a DAP or a lysine residue to obtain peptides 3-7. To remove the benzyl group, following the cleavage from the resin as described, the lyophilized peptide was dissolved in anhydrous DCM and treated in an ice bath with excess BCl_3 (1 M solution in DCM) over a stream of nitrogen. The resulting mixture was allowed to slowly warm to RT over nitrogen and then stirred overnight. Excess methanol was added to quench the reaction and stirring continued for one hour. The resulting solution was evaporated under reduced pressure, further dried under high vacuum for three hours, dissolved in a minimal amount of methanol and the product precipitated in ice-cold diethyl ether. Following centrifugation, the pellet was washed several times with diethyl ether, and finally dissolved in 50% ACN in water containing 0.1% TFA and freeze-dried for twenty-four hours. Finally, the peptide was purified by semi-preparative HPLC as described above. At the above-described BCl_3-mediated benzyl deprotection stage, for peptide 7 an acid-catalyzed esterification of the glutamic acid carboxyl group occurred, producing the methyl ester analogue 6. A portion of the methyl ester peptide 6 was hydrolysed back to compound 7 by raising the pH of a buffered solution of 6 to pH 9.5 and stirring and monitoring the hydrolysis rate via RP-HPLC. Finally, the peptide was purified by semi-preparative HPLC as above. For the preparation of peptide 8, the methylated iron chelator 2 was selectively conjugated to the peptide-resin at the C-terminal DAP residue using PyOxP/DIPEA chemistry as described above. Following cleavage from the resin, no deprotection of the chelator was performed in order to utilize such peptide as a non-chelating/control peptide.
For the assembly of peptide 11 and 12, the C-terminal Boc-Glu(OFm) group was treated with 20% piperidine to selectively remove the side-chain Fmoc group from the glutamate residue. Subsequently, the resin was treated with four-fold excess PyOxP and eight-fold excess DIPEA for five minutes followed by addition of the protected iron chelator 9 (for peptide 11) or the iron chelator 10 (for peptide 12) in equimolar amount to PyOxP. The mixture was shaken overnight, and the resin washed, dried and cleaved as described. For peptide 11, a BCl₃ treatment was performed to remove the benzyl group, whereas peptide 12 was directly purified via HPLC.
**Fig. S2:** Analytical RP-HPLC of the peptides (281 nm).

**Table S1:** HRES-MS of the compounds synthesized.

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/z found for [M+H]^+ (calculated)</th>
<th>m/z found for [M+2H]^{2+} (calculated)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>363.0904 (363.0981)</td>
<td>N/O</td>
</tr>
<tr>
<td>2</td>
<td>287.0661 (287.0668)</td>
<td>N/O</td>
</tr>
<tr>
<td>3</td>
<td>831.4265 (831.4266)</td>
<td>416.2169 (416.2172)</td>
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<tr>
<td>4</td>
<td>789.3803 (789.3796)</td>
<td>395.1939 (395.1938)</td>
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<tr>
<td>5</td>
<td>808.3528 (808.3531)</td>
<td>404.6801 (404.6804)</td>
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<td>6</td>
<td>846.3914 (846.3899)</td>
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<tr>
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<td>N/O</td>
</tr>
<tr>
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<td>783.4260 (783.4266)</td>
<td>392.2166 (392.2172)</td>
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<tr>
<td>12</td>
<td>783.4259 (783.4266)</td>
<td>392.2165 (392.2172)</td>
</tr>
</tbody>
</table>

*Not Observed*

**Fluorescence quenching/dequenching assays**

**Cell-free system:** A 100 μM Fe-NTA solution in MOPS, pH 7.4 was prepared. The probe concentration was 20 μM in 50 mM MOPS, pH 7.4. In independent cuvettes, to 1.5 mL MOPS buffer an appropriate volume was withdrawn and an identical volume was added from the 100 μM Fe-NTA solution to give a final desired Fe-NTA concentration; to this solution 1.5 mL aliquots of the probe solutions were added to yield a constant 10 μM final probe concentration. Fluorescence emission spectra were then acquired for each sample to determine the extent of quenching upon probe/Fe-NTA incubation.

**In-cell studies:** Responsiveness of fibroblasts FEK4 cells to iron was achieved by loading them with iron(III)-8-hydroxyquinoline [Fe(HQ)_3] which was prepared freshly by mixing a solution of ferric chloride (10 mM) in water with 8-hydroxyquinoline (30 mM) in DMSO so as to obtain a Fe:ligand ratio of 1:3. The complex was then allowed to form for 1 h at 37 °C before use on cells. Briefly, FEK4 cells were first incubated (or not) overnight, with 50 μM compound 3. The next day, the medium was removed, the cells washed twice with PBS, trypsinized, resuspended in buffer F (10 mM HEPES pH 7.3, 150 mM NaCl) and counted. 10⁶ cells were transferred to a thermostated (37 °C) quartz cuvette and kept in suspension for fluorescence reading with a Kontron spectrofluorimeter (SFM 25, Eching, Germany). Fluorescence was monitored until a stable signal was obtained, upon which iron was added in 1.5 μM increments as Fe(HQ)_3 complex and fluorescence monitored until a stable signal was obtained.
obtained before the next addition of iron. Dequenching of fluorescence was achieved by adding deferiprone (an initial 10 \( \mu \text{M} \), followed by 20 \( \mu \text{M} \) increments) to the iron-loaded cells. PBS, buffer F and the quartz cuvette were all chelex-treated so as to avoid unwanted quenching of compounds’ fluorescence.

**pKa and Iron stability constants**

The automatic titration system used in this study comprised an autoburette (Metrohm Dosimat 765 liter mL syringe) and Mettler Toledo MP230 pH meter with Metrohm pH electrode (6.0133.100) and a reference electrode (6.0733.100). 0.1 M KCl electrolyte solution was used to maintain the ionic strength. The temperature of the test solutions was maintained in a thermostatic jacketed titration vessel at 25°C ± 0.1°C by using a Techne TE-8J temperature controller. The solution under investigation was stirred vigorously during the experiment. A Gilson Mini-plus#3 pump with speed capability (20 mL/min) was used to circulate the test solution through a Hellem quartz flow cuvette. For the stability constant determinations, a 50 mm path length cuvette was used, and for pKa determinations, a cuvette path length of 10 mm was used. The flow cuvette was mounted on an HP 8453 UV-visible spectrophotometer. All instruments were interfaced to a computer and controlled by a Visual Basic program. Automatic titration and spectral scans adopted the following strategy: the pH of a solution was increased by 0.1 pH unit by the addition of KOH from the autoburette; when pH readings varied by <0.001 pH unit over a three second period, an incubation period was activated. For pKa determinations, a period of one minute was adopted; for stability constant determinations, a period of five minutes was adopted. At the end of the equilibrium period, the spectrum of the solution was then recorded. The cycle was repeated automatically until the defined end point pH value was achieved. All the titration data were analyzed with the pHab program. The species plot was calculated with the HYSS program. Analytical grade reagent materials were used in the preparation of all solutions.
Fig. S3: A: Spectrophotometric titration of $4 = 7.2 \mu M$ starts in 20.249 mL 0.1 M KCl, pH from 1.822 to pH 11.034. B: Speciation of $5$ over pH 2-11. C: Experiment with $5 = 163 \mu M \ [Fe^{3+}] = 40.8 \mu M$ ratio of L:M= 4, starts in 20.421 mL 0.1 M KCl, pH from 1.551 to pH 10.997. D: Speciation plot of $4 = 10^{-5} M$ with $[Fe^{3+}]= 10^{-6} M$ over pH 0-12.

Fitting the pH-induced spectral changes (Fig. S3A) enabled the identification of 3 species (Fig. S3B). Titration in the presence of iron(III) (molar ratio 1:4) led to the identification of 3 iron complexes, FeL, FeL$_2$ and FeL$_3$. The latter complex dominates in the pH range 6-10.

Live cell microscopy

Subcellular localization of the peptides was analysed as previously described$^6$, using an Olympus IX51 inverted epifluorescence microscope equipped with a 100 W mercury UV lamp. Cells were imaged with a 40× objective. Images were acquired via an Olympus DP72 digital camera controlled by Olympus cell’P Analysis Image Processing software (Soft Imaging System GmbH). Cells showing yellow staining, resulting from superimposition of the peptide-specific green signal and the organelle-specific red signal, were qualitatively considered positive for co-localization. The extent of co-localization of each compound with mitochondria was also measured quantitatively by Manders’ correlation coefficients (MCCs) using ImageJ software with the JaCoP plug-in$^7$, from a random selection of image fields. MCCs provide a measure of how much of the signal intensity of a channel occurs in the same location as the other channel. A bar chart is provided as Fig. S5, depicting values obtained for MCC M1, which represents the extent of overlap of compound signal (green) with the organelle signal (red). Values above 0.5 indicate significant co-localisation of the mitochondria-targeted green probe with the mitochondria red marker. Finally, analysis of intensity profiles (e.g. Fig. S4b) collected across cells (over a length of ca 40-50 µm) using cell’P Image Processing software confirmed the conclusions obtained from both qualitative and quantitative analyses of microscopy pictures.
Fig. S4: Representative microscopy images of intracellular co-localization studies of peptides 5 (A-D), 11 (E-H) and 12 (I-L) with mitochondrial compartments (a). Representative pixel intensity
profiles collected across field sections (indicated by white arrows) of two-colour images of compounds 3 and 5 (panels D of Figure 4 and S4 respectively) are also shown (b). Note the marked overlap of the profiles. Green profiles depict fluorescent signals from the compounds tested, whereas red profiles account for fluorescence from mitochondrial marker.

**Fig. S5:** Quantitative analysis of co-localization.
The extent of co-localization of each compound with mitochondrial compartments was measured quantitatively by Manders’ correlation coefficient M1 using ImageJ software with the JaCoP plug-in as described in Live cell microscopy section. M1 represents the fraction of signal from the compound co-localizing with signal from the organelles. The values indicated show the means +/- SD of M1 per field, corresponding to evaluation of at least two random fields of 8-10 cells over three independent experiments.

**Fig. S6:** Cytotoxicity assay of the peptide-chelators.
FEK4 cells were incubated with the peptide-chelators indicated in the conditions used for live cell microscopy (Fig. 4 and S4) (i.e. 50 µM in phenol red-free conditioned medium, overnight at 37degC), as well as 100 µM. The compound was then removed, replace with conditioned medium and viability
monitored by MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 24, 48 and 72h post-exposure to compound. The data present the MEAN and SD of three independent experiments.

Fig. S7: Compound 3 uptake by FEK4 cells. An example of chelator-peptide uptake by FEK4 cells is given. Cells were incubated overnight with increasing concentrations (0, 5, 10, 50 and 100 µM) of compound 3 and uptake by cells measured spectrofluorimetrically (instrument model CLARIOstar®, BMG labtech), λ<sub>ex</sub> 370 nm, λ<sub>em</sub> 470 nm.

References: