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Synthesis and biological evaluation of lipophilic iron chelators as protective agents from oxidative stress†

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Lipophilic Fe^{III} chelators were synthesized and shown to protect oligodendrial cells from oxidative damage induced by Fe^{III} and hydrogen peroxide.

Iron is a crucial element for fundamental cell functions, including the regulation of oxygen metabolism, the mediation of electron transfer processes, and for the biogenesis of complex molecules such as DNA and RNA.[1,2] Excess levels of free iron, however, are associated with severe neurological disorders such as Parkinson's disease, Friedrich's ataxia, Alzheimer's and Pica.[3–6]

The damaging effect of excess iron has been correlated to oxidative stress through the classical Fenton reaction, where Fe^{II} oxidizes H_2O_2, leading to the generation of hydroxyl radicals which are highly cytotoxic. DFO (desferrioxamine) is a natural iron chelator (isolated from *Streptomyces pilosus*) that is currently used to treat iron overload. Although it binds Fe^{III} with a high affinity, its slow onset of action, poor cell permeation and prolonged parenteral administration of extensive dosages have prompted the search for more effective iron chelators.[7–18]

In this communication, we present a novel class of lipophilic iron chelators (Fig. 1) that have a relatively low molecular weight; these compounds are potential drugs capable of crossing the blood brain barrier. Achieving cell permeation may, in turn, reduce the free Fe^{III} pool, shifting the cellular equilibrium $Fe^{II} \rightleftarrows Fe^{III}$ towards Fe^{III}, thereby reducing the level of Fe^{II} free to participate in the Fenton reaction. In addition, such chelators, based on the hydroxamic acid bi-dentate ligand, may diminish the toxic effect of reactive oxygen species (ROS), such as hydroxyl and superoxide radicals,[19–20] by generating relatively stable nitroxyl radicals.[21]

We shall demonstrate that the most potent analog (**5**) has a 25-fold higher protective effect in oligodendrial cells that were exposed to oxidative stress (Fe^{II} and H_2O_2) in comparison to DFO. To achieve these properties, we used an isopentyl group as a dipodal anchor, shown in related studies to assist in membrane crossing.[22–23] In addition, binding Fe^{III} in an octahedral geometry requires the looping of one of the strands on the dipodal anchor towards the binding cavity (Fig. 1). We have attained this geometry by exploiting a folding motif taken from collagen, introducing a proline–amino acid or amino acid–proline dipeptide prior to the external hydroxamate. The synthesis of these iron chelators was carried out by coupling *O*-benzyl hydroxylamine to one of the carboxylic acids of the symmetrical dipod,[24] then coupling an ester-extended *O*-benzyl hydroxylamine[25] to the second pod, affording a non-symmetrical compound **1** with a dipodal anchor. Compound **1** was finally coupled to dipeptides **2**–**4** with an external hydroxamic acid group (Fig. 2), providing the final compounds **5**–**7** (Fig. 1) which

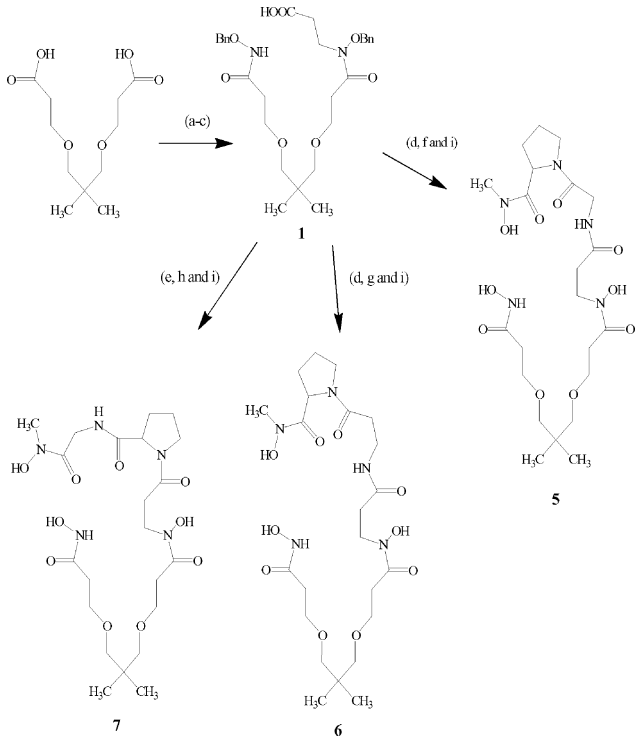

Fig. 1 Synthesis of the iron chelators: (a) EDC, *O*-benzyl hydroxylamine; (b) DCC, HOBT, $NH(OBn)CH_2CH_2COOEt$; (c) NaOH, MeOH; (d) DIC, HOBT, PCP; (e) **2**, DCC, HOBT; (f) **3**, NEt_3; (g) **4**, NEt_3; (h) TFA; (i) H_2, Pd/C, EtOH.

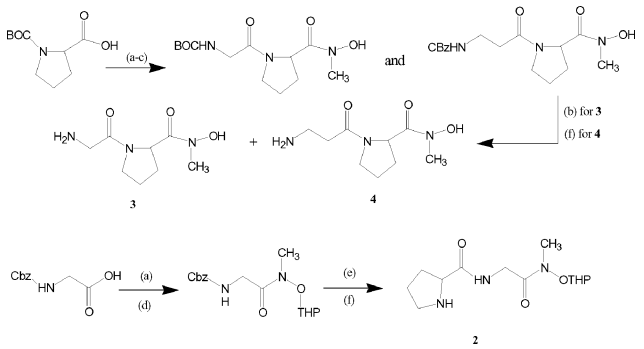

Fig. 2 Synthesis of dipeptides containing the hydroxamix acid group: (a) *N*-methyl hydroxylamine, TMSCl, NMM, isobutyl chloroformate; (b) TFA; (c) BOC–Gly or CBz–β-Ala, DIC, HOBT; (d) DHP, TsOH; (e) H_2, Pd/C, EtOH; (f) Cbz–L-Pro, DMTMM.

were fully characterized by NMR, IR and MS. Fe^{III} titration of compounds **5**–**7** point to the formation of 1 : 1 metal to ligand complexes, as verified by MS and UV-VIS spectroscopy (see supporting information).

† Electronic supplementary information (ESI) available: Compound characterisation data, Fe^{III} titration curves. See http://dx.doi.org/10.1039/b507385p

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To investigate the protective abilities of these chelators, a spontaneously transformed cell line of oligodendroglia origin (OLN 93) was chosen as a model for neural cells. These cells were previously used to demonstrate a remarkable sensitivity to genotoxic stress, culminating in cell death when both divalent iron and H_2O_2 were added to cells.[26] In the present experiments, OLN 93 cells were seeded in 96-well polyethyleneimine pre-coated plates, and after 24 h attachment were further incubated for 3 h with the synthetic analogs at various concentrations. A Fenton reaction (50 μM Fe^{II} and 0.75 mM H_2O_2) was initiated, and after 2 h reagents were removed, and at designated times cell survival then measured by a neutral-red assay.[27] The survival rate is directly proportional to lysosomal dye uptake as measured by the absorbance at 550 nm.[28]

Fig. 3 demonstrates the protective effect of analogs **5**–**7** at a 200 μM concentration of chelator. Each data point is an average of seven wells. It is clear that all analogs exert some protective effect by increasing the overall survival rate of cells treated with H_2O_2 and Fe^{II}. We decided to focus on analog **5**, which displayed the highest protective effect (87% survival rate) of all three analogs.

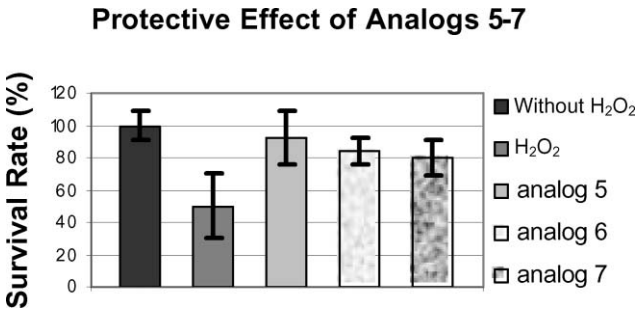

Fig. 3 The protective effect of analogs **5**–**7** as determined by a neutral-red assay. Cells were treated with Fe^{II} (50 μM) and H_2O_2 (750 μM) in the presence of 200 μM of chelator.

Fig. 4 presents a concentration gradient of analog **5**. Some apparent protection is observed at 40 μM concentration of chelator, and close to unity protection is observed at a concentration of 200 μM.

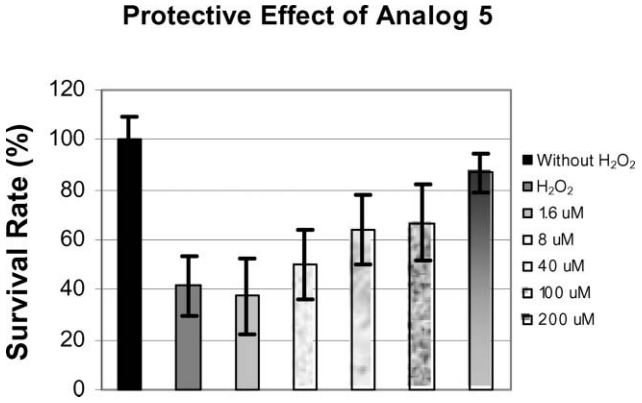

Fig. 4 Survival rate of OLN cells, after oxidative stress, at a varying concentration of analog **5**.

In order to determine whether this effect is unique to these compounds, we compared the most potent analog (**5**) to DFO (Fig. 5). While analog **5** almost fully protects the cells from oxidative stress (77% increase in cell survival), it is clear that DFO has a negligible effect (7.5% increase in cell survival). We attribute these differences to the lipophilic character of these synthetic analogs in comparison to the relatively hydrophilic character of DFO.[29]

It is unlikely that the observed protection of analogs **5**–**7** in comparison to DFO is based solely on Fe^{III} binding, as the

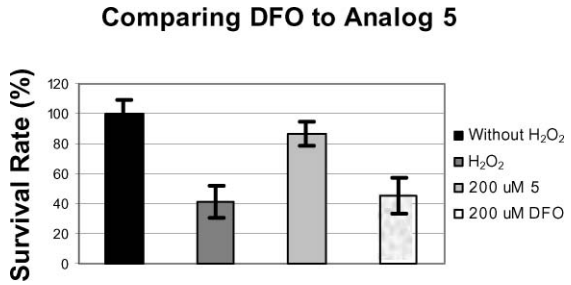

Fig. 5 Comparing the survival rate of OLN cells incubated with 200 μM DFO or analog **5** after oxidative stress.

binding constants of all compounds are expected to be similar. Instead, we suggest that the lipophilic character of these analogs is the key element, allowing these compounds to cross the cell membrane, and so protect the cells from oxidative stress.

In conclusion, we have presented a series of Fe^{III} chelators that all show a protective effect on oligodendrial cells that were exposed to Fe^{II} and H_2O_2. One analog (**5**) showed profound protection and was far more effective than the commercially available Fe^{III} chelator, DFO. Current efforts are directed toward improving the lipophilic character of these analogs by introducing hydrophobic side chain groups on the amino acids. Such modifications are expected to improve the chelators' activity by lowering the required dosage for effective protection.

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27 Experiments were conducted on 96-well plates, providing seven repeats for each treatment. Error bars in Fig. 3–5 were calculated by standard deviation from average of these multiple repeats. Cells were incubated in Locke medium with 50 μM Fe^{II} for 3 h at 37 °C. H_2O_2 was added (0.75 mM) and incubated for 2 h at 37 °C. The medium was discarded and replaced by a DMEM medium (with antibiotics and 1% serum) and the cells were incubated at 37 °C overnight. The medium was discarded and a Neutral Red medium was added to the wells for 90–180 min. Neutral Red reading was performed on an Elisa reader and survival rates were normalized to wells that were not treated with H_2O_2.
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29 Partition coefficients of analog **5** and DFO (as free ligands) were determined in water–octanol as 14.5 and 0.8, respectively.