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A potent antioxidant small molecule aimed at targeting metal-based oxidative stress in neurodegenerative disorders†

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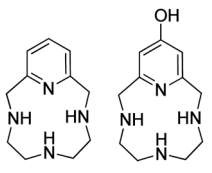
Metal-ion misregulation and oxidative stress have been linked to the progressive neurological decline associated with multiple neurodegenerative disorders. Transition metal-mediated oxidation of biomolecules *via* Fenton chemical reactions plays a role in disease progression. Herein we report the synthesis, characterization and antioxidant activity of 2; a pyclen derivative with enhanced antioxidant character.

Oxygen plays a vital role in maintaining the energy metabolism of living organisms.[1] In fact, the brain comprises 2% of the total body mass and consumes about 20% of total body oxygen. Neurons and astrocytes are responsible for the brain's enormous use of oxygen, as it is utilized in cellular respiration and for the biosynthesis of neurotransmitters.[1,2] Due to its elevated oxygen demand, the brain can become susceptible to oxidative damage, induced by unregulated redox-active metals such as Fe and Cu.[2–5] Reactions of free Cu or Fe with oxygen and a biological reductant such as ascorbate, can lead to an excess production of reactive oxygen species (ROS) *via* Fenton chemical reactions giving rise to oxidative stress.[6,7]

Transition metal ions such as Cu, Fe and Zn are essential for neuronal functions that involve free radical detoxification, electron transport, oxygen transport, neurotransmitter biosynthesis and neurotransmission.[5,8,9] Disruptions or alterations in metal-ion regulatory pathways could contribute to an excessive build-up of ROS; known to cause protein aggregation, free radical generation and oxidative stress.[10,11] Oxidative stress, coupled with transition metal ion misregulation has been implicated in multiple neurological diseases to date which include: Huntington's, Alzheimer's (AD), Parkinson's (PD) and Lou Gehrig's disease.[5,11] In AD increased levels of Cu, Fe and Zn accumulate in the beta-amyloid plaque affected regions of the brain, such as the hippocampus, and these regions display increased levels of oxidative injury.[3,10] Furthermore, in PD total Fe levels are increased specifically in the substantia nigra, along with a 30–40% decrease in glutathione concentration.[10,11]

Using the rationale that oxidative stress, in conjunction with metal ion misregulation are key factors in neurological disease development, current therapies for these disorders are aimed at disrupting the aberrant metal ion chemical reactions that produce excess ROS.[2,12,13] Treatment with Vitamin E and coenzyme Q10 in animal models of AD and PD, respectively, were found to be neuroprotective but display no proven benefit in the clinical setting.[1,10,14] These results are indicative of the need for a small molecule containing both ionophore and antioxidant activity.[15,16] A small molecular system capable of bimodal modulation of the metal-ions, as well as regulation of ROS would prove useful in combating these disorders.

Previous studies have shown that tetraazamacrocycles are capable of disrupting metal-induced beta-amyloid formation; in addition, we recently showed that **1**, (Chart 1), possessed antioxidant and protective capacity against ROS induced cell death.[5,12,14,17] Given this success we set-out to enhance the antioxidant activity of **1**, without affecting the metal ion chelation capability.[18,19] The conversion from a pyridine to a pyridol, (**2**) was pursued as pyridols are known to react with hydroxyl radicals *via* addition at the C3 or C5 position of the pyridol ring with the products being reminiscent of tannins, well known for their antioxidant power.[3,18,20,21] Methods used



1 2

Chart 1

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† Electronic supplementary information (ESI) available: Synthesis and characterization of **2**, assay protocols and supporting figures. CCDC 905445 and 911804. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2cc36808k

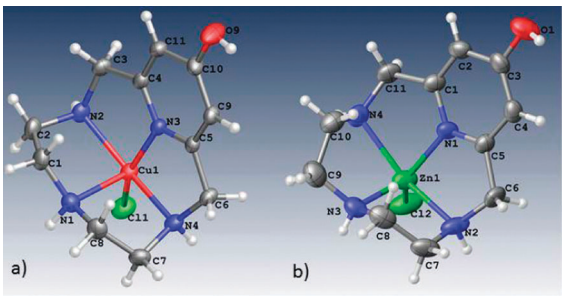

Fig. 1 Thermal ellipsoid plots at 50% probability of (a) [Cu(**2**)·Cl]$^+$ and (b) [Zn(**2**)·Cl]$^+$. The perchlorate ion is removed for clarity.

to produce **2** are detailed in Schemes S1–S3 (ESI†). Reaction of **2**, at pH 7, in water with either Cu(ClO_4^-)$_2$ or Zn(ClO_4^-)$_2$ readily yielded crystals suitable for X-ray diffraction analysis. As shown in Fig. 1, the coordination environment is similar to the Cu^{II} and Zn^{II} congeners reported for **1**; as the ligand bends producing a square pyramidal coordination around the metal cation, with a chloride ion coordinated in the fifth axial position to the metal ion.[22] The Cu^{II} and Zn^{II} complexes are bent 112° and 137° with respect to the planes formed by the N-donors and metal-ions. These measurements along with other bond lengths and angles are consistent with other N-heterocyclic complexes with these ions. A full comparison of the XRD data can be found in the supplemental section (Tables S1 and S2, ESI†).

Redox active metal ions produce a variety of species known to cause cellular damage including radicals and superoxide. As such, the capability of **2** to reduce radicals was investigated using the radical DPPH, (2,2-diphenyl-1-picrylhydrazyl). DPPH is a stable radical in solution having an absorbance maximum at 520 nm. As DPPH is reduced, the absorbance intensity of the solution decreases, along with a change in color from purple to yellow. As shown in Fig. 2, the pyridol congener of **1** displays the ability to reduce free radicals in solution at concentrations ranging from 1000–100 μM; meanwhile, displaying greater radical reducing ability at higher concentrations. Based on these results, we can attribute the radical reducing functionality of **2** to its hydroxyl group, as this feature is lacking in **1**.

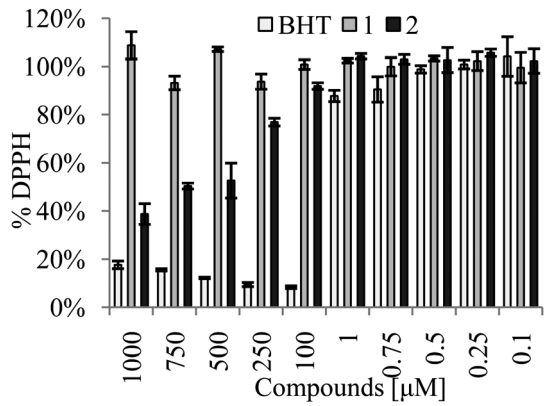

Fig. 2 DPPH radical quenching assay showing the antioxidant character of **1** and **2** compared to the standard antioxidant BHT. n = 3. BHT = butylated hydroxyl toluene (positive control).

Furthermore **1** and cyclen‡ lack the capability to reduced DPPH, as these absorbance values were equivalent to negative control (95% EtOH) levels.

Cyclic voltammetry was then performed to confirm that **2** helped mitigate the redox behaviour of Cu^{II}.[22] Fig. S1 (ESI†) compares data obtained for the perchlorate salts of Cu(**1**)·Cl and Cu(**2**)·Cl, $E_{1/2}$ = −602 and −651 mV, respectively. The pyridol group results in a shift of the Cu(II/I) couple to a slightly more negative value than Cu(**1**) indicating the presence of the hydroxyl group is slightly electron donating. These potentials are 400 mV more negative than those expected to redox couple with biological reductants such as ascorbate.

Under biological conditions oxygen in the presence of ascorbate and copper is catalytically reduced to a hydroxyl radical; which can be detected by co-incubation with CCA, to produce the fluorescent hydroxy-CCA species. We performed this reaction *in situ* and showed that this process was halted by the addition of **2** along with structurally similar chelates as well (Fig. S2, ESI†), presumably by chelation of the copper resulting in a shifting of the Cu$^{II/I}$ potential outside the ascorbate window as shown by Faller and co-workers.[7,23] These *in situ* studies show that **2** can counter-act two components involved in oxidative stress: radicals and metal-ion redox cycling.

Next, cellular studies were carried out to evaluate the intracellular antioxidant efficacy of **2** compared to **1**, using the cell-permeable fluorophore 2,7′-dichlorodihydrofluorescin diacetate, (DCFH-DA), as an indicator for ROS. DCFH-DA diffuses into cells and is deacetylated by cellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescin, (DCFH), which is subsequently oxidized to the highly fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCF), in the presence of ROS. The fluorescence intensity is directly proportional to the amount of ROS present in cell cytosol. FRDA cells (Friedreich's Ataxia Fibroblasts) were utilized in this experiment, since these cells have higher levels of cytosolic ROS resulting from mitochondrial malfunction; therefore, serve as a good model for studying metal induced oxidative stress.[24] BSO (2-amino-4-(butylsulfonimidoyl)-butanoic acid) was used to induce oxidative stress in the cells, as it inhibits the synthesis of glutathione in cells.

The results of the DCFH-DA cell culture assay indicate that **1** is an effective antioxidant in the 1.25 nM–1.25 μM range. As shown in Fig. 3, compound **2** is a potent antioxidant at each of the concentrations tested from 1.25 nM to 1.25 μM. While **1** is capable of quenching the ROS induced by BSO, **2** also inhibits the naturally occurring ROS present when compared to non-treated cells (media only). Fig. 3 shows at concentrations of 1.25 nM, 12.5 nM, 125 nM and 1.25 μM for **2**, there is a decrease of 33%, 58%, 75%, and 87% [ROS], respectively, compared to the control (media only). Again, we see that the antioxidant activity of **2** increases with concentration, consistent with the results from the DPPH study. Our studies indicate that the binding of metals-ions to **2** is quite similar to **1**, therefore the enhanced antioxidant activity is attributed to the pyridol component. Preliminary NMR data suggest –OH addition onto the pyridol ring when **2** is incubated with H_2O_2.[19] Currently we are working to fully characterize the product(s) of 2 upon reaction with Fe^{II} and H_2O_2 (Fenton reaction).

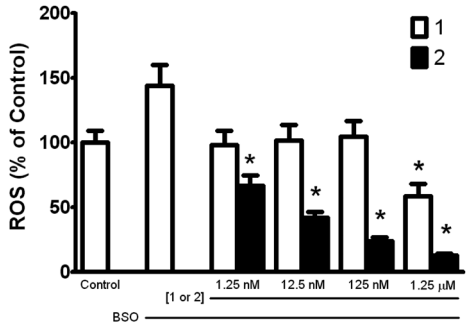

Fig. 3 DCFH-DA fluorescent response in FRDA cells after 12 hours exposure to BSO [1 mM] showing dose dependence with **1** and **2**. n = 8. *Indicates significance with respect to BSO control and a p value < 0.05.

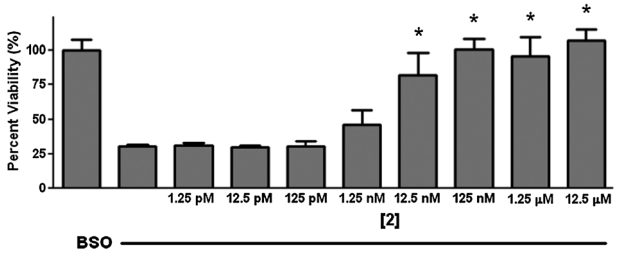

Fig. 4 Calcein AM viability assay of FRDA cells after 48 hour exposure to BSO [1 mM] followed by addition of **2**. n = 8 for each sample. *Indicates significance with respect to BSO control and a p value < 0.05

Preliminary ^{13}C NMR data suggest electrophilic –OH addition onto the pyridol ring, when **2** is incubated with H_2O_2; consistent with similar compounds reported in literature.[18–20]

Finally, cell viability studies using live-cell penetrating Calcein AM as a fluorophore show that **2** protects cells against BSO insult ranging from 1.25 nM–12.5 μM, with an EC50 31.46 ± 4.96 nM (Fig. 4). This dose response is visualized in the fluorescent cell images, Fig. S3 (ESI†), which show that cell viability increases with an increasing concentration of **2**. An interesting feature to be noted from these cellular studies is the fact that both **1** and **2** are capable of entering cells and do not appear to interrupt the vital functions of cytosolic metalloenzymes, presumably *via* metal ion extraction.

Finally, the macrocyclic compound **2** is a chelator capable of preventing metal-induced amyloid formation as well as disaggregation as evidenced by Turbidity and Tyr-10 fluorescence studies which largely parallel the activity observed with **1** (Fig. S4–S6, ESI†). These studies are consistent with the reactivity of former chelating systems reported by others as well.[25–27]

In conclusion, a new small molecule **2** has shown potential as a metal-binding and potent antioxidant agent. The antioxidant capacity of **1** was enhanced dramatically *via* conversion of the pyridine backbone to a pyridol, **2**. We have shown that this compound is capable of reducing stable radicals, such as DPPH and halts $Cu^{II/I}$ redox cycling responsible for the production of ROS. Cellular studies showed that **2** has a marked improvement of preventing cellular death, induced by oxidative stress.

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Notes and references

‡ Pyclen = 1,4,7,10-Tetraaza-2,6-pyridinophane, Cyclen = 1,4,7,10-Tetraazacyclododecane, Hydroxylpyclen = 3,6,9,15-Tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-trien-13-ol, DCFH = Dichlorodihydrofluorescein, BHT = Butylhydroxytoluene, DPPH = 2,2-Diphenyl-1-picrylhydrazyl, BSO = Buthionine sulphoximine, CCA = Coumarin-3-carboxylic acid, FRDA = Friedreich's ataxia.

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