Altering pyridinone *N*-substituents to optimise activity as potential prodrugs for Alzheimer's disease†

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Selective design modifications of specifically substituted 3-hydroxy-4(1*H*)-pyridinones show possibly advantageous ring freedom while maintaining metal-binding ability and antioxidant capacity, moving toward an efficient potential treatment for Alzheimer's disease.

The recent success of metal-attenuating compounds (now in phase 2 clinical trials) for the treatment of Alzheimer's disease (AD) has brought further support to the amyloid cascade hypothesis and emphasises the role that cerebral metals (Cu, Fe and Zn) play in the pathology of AD.[1,2] Metal-binding pyridinone prodrugs (Fig. 1) that possess antioxidant characteristics and brain targeting abilities have been suggested as treatments for AD that may have better efficacy than treatments that only sequester metals.[3]

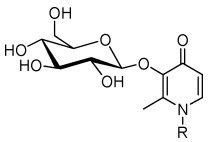

Fig. 1 Generic structure of a glycosylated pyridinone prodrug as a potential multifunctional AD therapeutic.[3]

Pyridinones are chosen as the scaffold for elaboration because they are generally non-toxic, and approved for therapeutic use in many parts of the world (including Europe, where 3-hydroxy-2-methyl-4(1*H*)-pyridinone is used to mitigate iron overload in thalassemia patients).[4] Hydroxypyridinones are effective metal binders ($\log\beta_2 = 20$–22 for Cu^{2+},[5] 11–18 for Zn^{2+};[6] and $\log\beta_3 = 27$–37 for Fe^{3+})[7] and their structure is easily modified by altering the *N*-substituent; which may be varied without significantly affecting the metal binding efficiency of the pro-ligand.[8]

Because the brain requires a large portion of the body's energy intake, it is often found that molecules incorporating a glucose moiety will be transported into the brain;[9] this targeting strategy has been used to effectively elevate brain uptake of compounds such as dopamine for Parkinson's disease therapy and opiate analgesics.[10,11] In our design strategy, this is achieved by attaching a glucose moiety at the 3-hydroxyl oxygen. Once in the brain, the glucose moiety can be enzymatically removed, leaving an efficient metal binding agent that will sequester metals through the α-hydroxy-keto functionality of the pyridinone moiety.[3] Among a range of other pyridinone compounds, 3-(β-D-glucopyranosyloxy)-2-methyl-1-phenyl-4(1*H*)-pyridinone (**Gppp**) has been previously synthesised in our group; enzymatic cleavage of the glycosidic bond was demonstrated using *Agrobacterium sp.* β-glucosidase.[12]

The medically interesting and testable forms of these compounds are the active, un-glycosylated species that are capable of binding metals. Members of our research group have proposed a series of pyridinones showing moderate activity in *in vitro* AD tests.[3] Our work has focused on optimising the pro-ligand structures to maximise their efficacy against the symptoms of AD. Two pyridinones that have been tested against some of the biomarkers of AD are shown in Fig. 2.

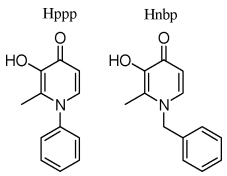

Fig. 2 Analogous hydroxypyridinones **Hppp** (left) and **Hnbp** (right).

3-Hydroxy-2-methyl-1-phenyl-4(1*H*)-pyridinone (**Hppp**) was first studied as a potential AD treatment because it incorporates the desired antioxidant capacity and metal-binding ability.[3] Adding a methylene spacer between the pyridinone ring and the phenyl ring (such as in **Hnbp**) may alter the antioxidant capacity and may also affect the ability to infiltrate and ameliorate toxic beta-amyloid (Aβ) deposits within the brains of AD patients.

Hppp was prepared by previously reported methods.[13] 1-Benzyl-3-hydroxy-2-methyl-4(1*H*)-pyridinone (**Hnbp**) was synthesised based on methods reported by Kitagawa *et al.*[14] and Barta *et al.*[15] All products were isolated as colourless solids; intermediate species were characterised using ^{1}H NMR spectroscopy, final products were additionally characterised by ^{13}C{H} NMR spectroscopy and elemental analysis, and **Hnbp** was further characterised by X-ray crystallography.

Demonstration of metal-binding ability was achieved through synthesis of metal complexes of **Hppp** and **Hnbp**. Copper complexes are of particular interest because copper is one of the redox-active metals involved in AD, and has higher affinity for Aβ than does iron.[16] In this light, copper complexes were synthesised by adding two equivalents of deprotonated pro-ligand to a solution of $Cu(ClO_4)\cdot 6H_2O$ in a mixture of dichloromethane and methanol. Stirring the mixture for 6 h afforded the copper complex as a fine

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† Electronic supplementary information (ESI) available: experimental details of syntheses, turbidity, TEAC and MTT assays; selected crystallographic data for **Hnbp**, Cu(**ppp**)$_2$ and Cu(**nbp**)$_2$. CCDC reference numbers 697416–697418. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b815404j

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Table 1 Selected crystallographic data for **Hnbp**·$CHCl_3$, Cu(**ppp**)$_2$·$2CHCl_3$ and Cu(**nbp**)$_2$·$2CHCl_3$

	Hnbp·$CHCl_3$	Cu(**ppp**)$_2$·$2CHCl_3$	Cu(**nbp**)$_2$·$2CHCl_3$
Formula	$C_{13}H_{13}NO_2$·$CHCl_3$	$C_{24}H_{20}N_2O_4Cu$·$2CHCl_3$	$C_{26}H_{24}N_2O_4Cu$·$2CHCl_3$
M	334.61	702.70	730.75
Crystal system, space group	Monoclinic, $P2_1/c$ (#14)	Orthorhombic, *Pbca* (#61)	Monoclinic, $P2_1/n$ (#14)
a/Å	11.7981(13)	11.5466(11)	6.3840(3)
b/Å	10.8093(13)	11.6975(11)	21.0223(12)
c/Å	12.5786(15)	20.9936(18)	11.7249(7)
β/°	107.760(6)	90	96.787(2)
V/Å^3	1527.7(3)	2835.5(5)	1562.53(15)
Z	4	4	2
T/K	173(2)	173(2)	173(2)
Refln collcd/unique	16 003/3665 ($R_{int} = 0.025$)	35 879/2789 ($R_{int} = 0.046$)	13 415/3545 ($R_{int} = 0.033$)
Residuals (F^2, all data)	w$R2 = 0.110$	w$R2 = 0.071$	w$R2 = 0.163$
Residuals (F, $I > 2\sigma(I)$)	$R1 = 0.045$	$R1 = 0.028$	$R1 = 0.053$

green precipitate; X-ray quality crystals were obtained *via* liquid–liquid diffusion of chloroform and diethyl ether.

Green prism crystals of Cu(**ppp**)$_2$ and Cu(**nbp**)$_2$ were analyzed using X-ray diffraction techniques (see Table 1) and assigned the structures shown in Fig. 3. These complexes co-crystallised with two molecules of chloroform per copper centre, which is not unknown for bis-pyridinonato–copper complexes crystallised in this manner.[17] The *trans* and nearly square planar coordination geometry that is seen in these complexes is also exhibited in the few other structurally characterised pyridinone–copper complexes.[5b,17,18] Comparing the solid-state structures of Cu(**ppp**)$_2$ and Cu(**nbp**)$_2$ we see that they are very similar in their coordination geometries; however, Cu(**nbp**)$_2$ possesses additional ring freedom compared to that found in Cu(**ppp**)$_2$.

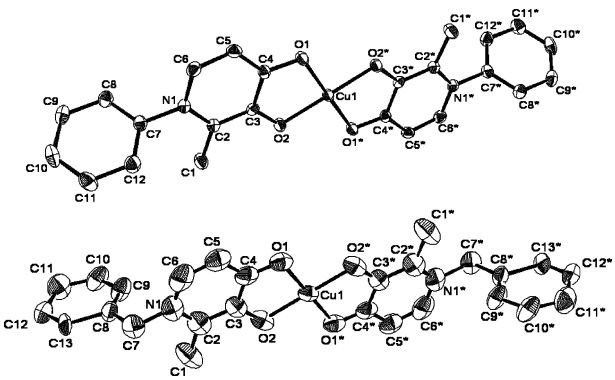

Fig. 3 Ellipsoid plots (50% probability, H-atoms omitted for clarity) of Cu(**ppp**)$_2$ (above) and Cu(**nbp**)$_2$ (below).

In the solved structure of Cu(**nbp**)$_2$, the benzyl ring is found in a variety of orientations within the crystal lattice; the structure shown in Fig. 3 is representative of one of these orientations. In the Cu(**ppp**)$_2$ complex, the phenyl ring is oriented at an angle of 63° relative to the pyridinone ring. The additional freedom of ring orientations seen in solid state Cu(**nbp**)$_2$ and presumably in **Hnbp** itself, may allow the compound to better infiltrate Aβ aggregates and permit improved access to the problematic metal species involved in AD.

It is unlikely that either of these pyridinones remain in a fixed-ring orientation while in physiological conditions, however the solid state structures hint that there is a smaller energy barrier to rotation with **Hnbp** which may prove to be advantageous. Further investigation is required to determine the magnitude of this effect *in vivo*.

A turbidity assay was performed to investigate the ability of **Hnbp** to dissolve aggregated Aβ species *in vitro*. Synthetic human $A\beta_{1-40}$ was dissolved in a buffered aqueous solution to which was added metal ions (Cu^{2+} or Zn^{2+}, in different trials). Copper and zinc were used in this assay as they are found in elevated concentrations in Alzheimer's amyloid plaques.[19,20] Metal ion addition causes Aβ aggregation, which, through formation of a turbid solution, is measureable by absorption spectroscopy; suspended solids (such as aggregated Aβ) scatter light and give an increase in apparent absorbance. Addition of metal binding pro-ligands reduces this apparent absorption and thus a comparison of Aβ disaggregating ability can be made between pro-ligands; lower absorbance indicates greater efficacy. Diethylenetriaminepentaacetic acid (DTPA) was used as a positive control; it is a hexadentate ligand known to bind metals and dissolve metal-containing Aβ aggregates.[3,21]

Hppp and **Hnbp** significantly reduce the amount of aggregated Aβ compared to the "Aβ and metal" negative control for both the copper and zinc trials (Fig. 4). In both metal trials, the abilities of **Hppp** and **Hnbp** to reduce Aβ aggregates in solution were found to be statistically equivalent.

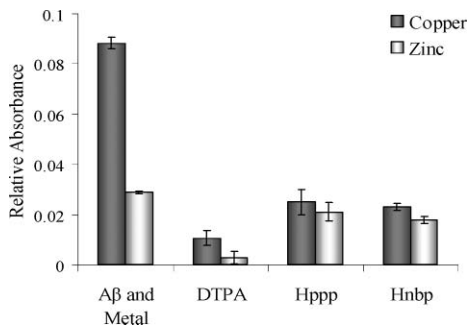

Fig. 4 Dissolving Aβ aggregates; absorbance taken at 45 min after addition of test pro-ligands to metal-aggregated Aβ. Bars indicate mean absorbance of solutions at 405 nm ($n \geq 3$) and error bars indicate ± standard deviation.

The antioxidant capacities of **Hppp** and **Hnbp** were determined using a trolox equivalent antioxidant capacity (TEAC) assay.[22] 2,2′-Azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) is easily made into a radical cation ($ABTS^{\bullet+}$) that absorbs strongly at 745 nm, but does not absorb at this wavelength in its neutral

and non-radical form.[22] Measuring the reduction of absorbance upon addition of a compound gives a value for the antioxidant capacity of that compound. The antioxidant capacities of **Hppp** and **Hnbp** are compared with that of α-tocopherol (vitamin E, a known antioxidant) in Fig. 5. For all test compounds, the TEAC was calculated by normalising the effective radical quenching of each test compound to that of trolox, at time points of 1, 3 and 6 min. At all time points, the TEAC values for **Hppp** and **Hnbp** are statistically equivalent, and are comparable to that of α-tocopherol.

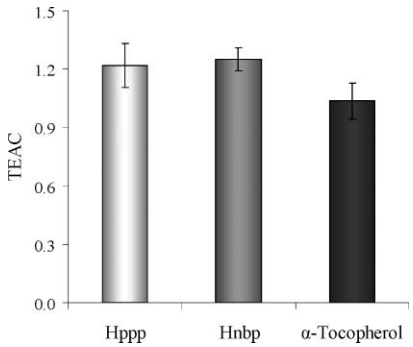

Fig. 5 Antioxidant activity of hydroxypyridinones **Hppp** and **Hnbp** compared to α-tocopherol (vitamin E) at the 6 min time point. Shown are means of three trials and error bars represent ± standard deviation.

To examine their suitability as potential therapeutics, **Hppp** and **Hnbp** were subjected to cytotoxicity trials by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with human hepatocytes (cell line HepG2). Cells were exposed to test compounds for 72 h and their IC_{50}, or fifty percent inhibitory concentrations, were determined (Fig. 6). Cisplatin was used as a positive control because it is a rather toxic drug that is commonly used for the treatment of various forms of cancer.[23]

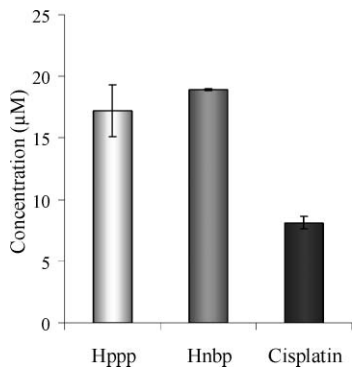

Fig. 6 IC_{50} values for pyridinone pro-ligands; represents concentration yielding 50% cell viability. Error bars indicate ± propagated error.

Hppp and **Hnbp** have comparable IC_{50} values (17 ± 2 and 18.9 ± 0.1 μM, respectively), and both are significantly less toxic than cisplatin (8.1 ± 0.5 μM), as expected. More hydrophilic *N*-substituents tend to provide pyridinones with higher IC_{50} values. More hydrophilic *p*-substituted-*N*-phenyl-pyridinones have been prepared with IC_{50} values in excess of 90 μM.[17] The performed assays have confirmed that the *in vitro* toxicity, antioxidant capacity and ability to dissolve Aβ aggregates are not significantly affected by the addition of a methylene spacer to our drug model. There is evidence from the solid-state structures of Cu(**ppp**)$_2$ and Cu(**nbp**)$_2$ that the addition of the methylene unit may allow more rotational freedom which may improve the compound's ability to infiltrate large Aβ aggregates *in vivo*; further investigation is called for.

The synthetic route utilised to produce **Hnbp** presents some synthetic advantages over other methods developed to synthesise pyridinones. It is thought that this synthetic method could be used to make a library of pyridinone pro-ligands similar to **Hnbp**, allowing this project to more fully examine the effect of *N*-substituent variation on pro-ligand activity.

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