Prodrugs

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Combating Alzheimer's Disease With Multifunctional Molecules Designed for Metal Passivation**

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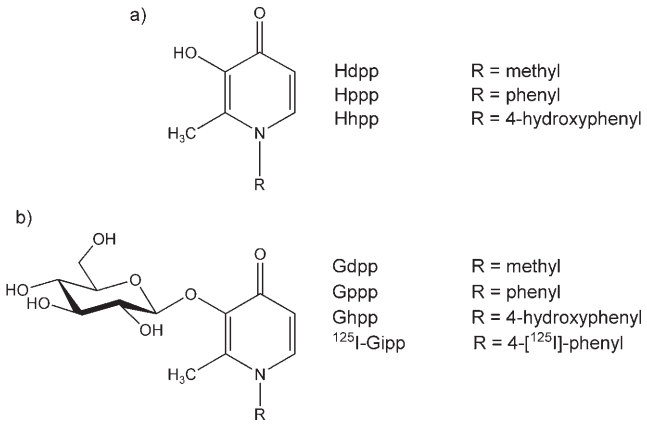
Two of the biochemical features of Alzheimer's disease (AD) that contribute to neurodegeneration are intracellular oxidative stress and elevated levels of trace metal ions, especially Fe^{III}, Cu^{II}, and Zn^{II}.[1] Both are factors involved in formation of the histological features in the brain used typically for postmortem diagnosis of AD, namely β-amyloid (Aβ) plaques and neurofibrillary tangles. Therapeutic interventions under current investigation elsewhere include clioquinol[2] and desferrioxamine,[3] which are metal chelators that target elevated trace-metal ions in the brain, although neither are intended to affect oxidative stress directly and nor are they targeted to the brain. Antioxidant supplements have been studied separately as palliative-only measures for alleviation of the symptoms of AD.[4]

Herein, we present for the first time a trifunctional approach to AD therapy. Modified and functionalized bidentate hydroxypyridinone pro-ligands (Scheme 1) address both the metal-ion and the oxidative imbalances inherent in AD while incorporating a glucose-receptor targeting feature.



Scheme 1. Hydroxypyridinones: a) nonglycosylated pro-ligands and b) their glycosylated prodrug forms designed for metal passivation in the brain as a therapeutic intervention in Alzheimer's disease (AD).

These prodrugs are designed to cross the blood–brain barrier (BBB), lose the pendant carbohydrate by enzymatic cleavage, passivate excess metal ions in the brain, and also protect neuronal cells against reactive oxygen species (ROS). Each of these functionalities has been demonstrated, thereby establishing the trifunctional principle as a valid goal in AD therapy. The prodrug strategy solves the potential problem of premature metal binding by using carbohydrates as both masking and directing substituents. In the context of increasing empirical support for re-establishing normal metal-ion homeostasis in neurodegenerative diseases, including AD, the trifunctional approach permits selective, tissue-dependent metal binding as a tailor-made, biologically compatible therapy.

To demonstrate the utility of this approach, a series of assays on prototype compounds have been undertaken, including both in vitro and in vivo studies. This strategy is aimed at reducing neurodegeneration from oxidative stress; by passivating the pro-oxidant metal ions Fe^{III} and Cu^{II}, the production of ROS can be expected to be lower. By changing the R group on the pyridinone ring, the aqueous solubility, lipophilicity, and BBB permeability can be modified. Prodrug hydroxy (OH) groups have been elaborated by glycosylation (Scheme 1 b) such that, after enzymatic deprotection, the free ligands will have ring OH groups available that can either efficiently trap radicals or bind metal complexes (Scheme 1 a). Removing metal ions that promote Aβ aggregation, such as Cu^{II} and Zn^{II}, also serves to prevent or reverse

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plaque formation.[1] The trifunctionality thus incorporates strong metal-binding, O-glycosylation to mask the chelator (preventing systemic metal binding), and antioxidant potential.

To show the feasibility of enzymatic removal of the pendant carbohydrate, we used a model system based on glycoside hydrolysis by a well-studied β-glucosidase from *Agrobacterium faecalis* (Abg; previously *Alcaligenes faecalis*) that has a relatively broad substrate specificity.[5] Abg was found to readily cleave glucose from the hydroxypyridinone glycosides (Scheme 1 b), as identified by thin-layer chromatography (TLC).

Hydroxypyridinones (a class that includes the structures shown in Scheme 1 a)[10] are well-known binders of di- and trivalent metal ions, with stability constants for Cu^{II} in the range of $\log\beta_2 = 20–22$[6] and 11–18 for Zn^{II}.[7] Considerably higher stability constants are observed for Fe^{III} ($\log\beta_3 = 27–37$).[8] Clioquinol, a Cu^{II} and Zn^{II} binder of intermediate affinity ($\log\beta_2$ (Cu) = 15.2; $\log\beta_2$ (Zn) = 12.47),[2b] recently completed successful phase II clinical trials and has been shown to dissolve Alzheimer-like amyloid deposits in transgenic mice.[2a] However, in comparison with our pro-ligands (Scheme 1), the 8-hydroxyquinoline is not designed to obviate systemic chelation, has known serious side-effects,[8] and is closely related to the gravimetric agent oxine, which is known to bind tightly to and precipitate a wide variety of di- and trivalent metal ions.[9]

After deprotection, the free ring OH groups can efficiently trap radicals. An improved 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation decolorization assay[11] was used to determine the relative Trolox Equivalent Antioxidant Capacity (TEAC) values of the compounds shown in Scheme 1 a. Note that the additional ring OH group in the phenol-derivatized hydroxypyridinone pro-ligand (Hhpp) promotes ROS scavenging, as measured by its $ABTS^{\bullet+}$ radical quenching ability. All of the compounds showed similar or higher TEAC values than vitamin E (α-tocopherol) and butylated hydroxytoluene (both common antioxidants), thereby demonstrating the potent antioxidant nature of these compounds (Figure 1).

The potential of these compounds to dissolve Aβ plaque was visualized turbidometrically. Aggregates of the $A\beta_{1–40}$ oligopeptide with Zn^{II} or Cu^{II} (pH 7.4 or 6.6 in HEPES buffer, respectively, HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) were exposed to representative prototype hydroxypyridinone pro-ligands (Scheme 1 a) in vitro and monitored with a modified turbidometric detection system in which the UV/Vis absorbance at 405 nm (A_{405}) was recorded.[12] Metal-associated aggregation of Aβ occurred upon addition of Zn^{II} or Cu^{II}, which was significantly attenuated upon subsequent additions of ligand solutions (Figure 2).

Brain uptake of a radiolabeled hydroxypyridinone glucoconjugate was assessed using an in situ rat brain perfusion technique.[13] ^{125}I-Gipp (shown in Scheme 1 b) in physiological saline was perfused for 60 seconds into the common carotid artery, the animal was terminated, and the brain was examined for radioactivity to determine tracer BBB permeability (see Supporting Information for further details).[14] The

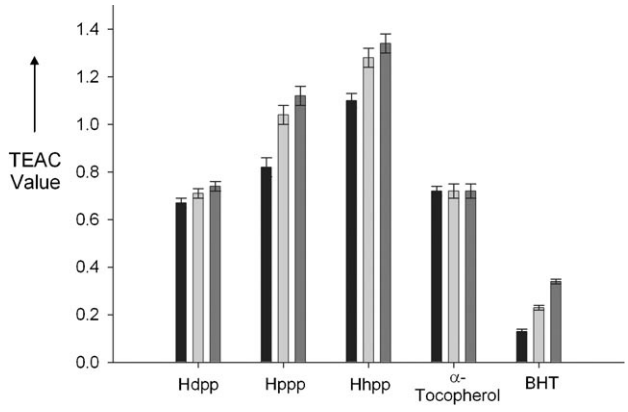

Figure 1. Antioxidant potential of nonglycosylated hydroxypyridinones compared to α-tocopherol and butylated hydroxytoluene (BHT) in a TEAC assay. TEAC data for 1 min are in black, for 3 min in light gray, and for 6 min in dark gray. Values represent the mean of three independent experiments (± standard deviation). See Supporting Information for further experimental details.

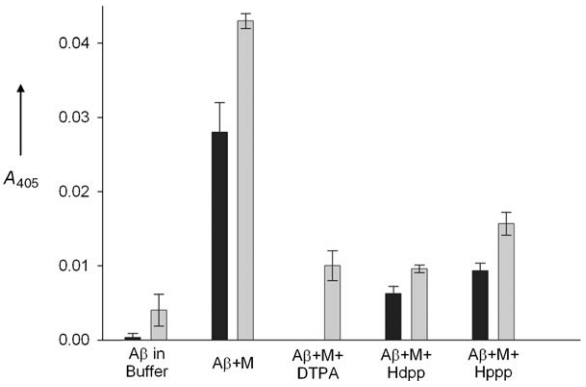

Figure 2. Comparison of ligands as inhibitors of Zn^{II}- or Cu^{II}-promoted Aβ aggregation, by turbidity measurements at 405 nm (A_{405}). Black bars indicate absorbance data from Zn^{II} trials at pH 7.4, while gray bars indicate data from Cu^{II} trials at pH 6.6. Final concentration of DTPA: 150 μM; Hdpp: 50 μM. Values represent the mean of at least three independent experiments (± standard deviation). DTPA = diethylenetriaminepentaacetic acid, a known strong metal ion chelator, is shown for comparison. See Supporting Information for further experimental details.

permeability of ^{125}I-Gipp was found to be 0.5 $\mu L\,s^{-1}\,g^{-1}$, similar to thiourea, although not as high as caffeine, theophylline, or ethanol (permeabilities determined previously).[15] BBB passage of the radiolabeled hydroxypyridinone compound signals adequate, although not stunning, cerebral uptake, which may be further enhanced by future ligand modifications.[16]

In summary, our results strongly support the designed mechanism of action in which our glycosylated prodrugs cross the BBB, release the active ligand by β-glucosidase-induced cleavage of the pendant carbohydrate, compete effectively for copper and zinc with the metal binding sites on Aβ peptide, and function as antioxidants within the central nervous system, thereby promising a new avenue for AD therapy. Unlike less specific biometal chelating compounds, the

specifically engineered compounds herein are intended not only to target metal ions after crossing the BBB, but also to lessen ROS in the brain, in a multifunctional strategy.[17] These trifunctional ligands combine metal sequestering and antioxidant properties with a third functionality, namely glucose conjugation, both to promote BBB permeability and to minimize systemic complexation of metal ions before the prodrugs are enzymatically cleaved to their active form.

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