Bis-8-hydroxyquinoline ligands as potential anti-Alzheimer agents†

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A strategy based on the use of a tetradentate ligand containing two linked 8-hydroxyquinolines allowed a significant increase in the ability to protect against β-amyloid peptide precipitation in the presence of Cu(II), Zn(II) and Fe(III) when compared to clioquinol, a 8-hydroxyquinoline derivative active *in vivo*.

Alzheimer's disease (AD) is a neurodegenerative disease associated with the aggregation of β-amyloid peptides (Aβ) in the brain.[1] Pathogenesis is linked to the formation of amyloid plaques, in particular from the highly amyloidogenic $A\beta_{1-42}$, whose production is fostered by AD-promoting mutations and risk factors. $A\beta_{1-42}$ has a high affinity for metal ions and forms insoluble aggregates, particularly in the presence of Cu(II), and to a lesser extent with Zn(II) and Fe(III). Moreover, the accumulation of redox active metal ions such as Cu(II) or Fe(III) in these amyloid structures is probably responsible for the generation of an oxidative stress, inducing brain neuronal lesions, which result in an irreversible loss of intellectual faculties. The redox activity of Cu(II)–Aβ can catalytically generate H_2O_2 in the presence of a reductant.[2]

Different metal ion chelators are able to dissolve amyloid aggregates.[3,4] Such a property suggests their possible use as therapeutic agents in the treatment of AD. In particular, clioquinol (Scheme 1) behaves as a metal ion chelator and dissolves amyloid plaques of the brain, probably by removing metal ions from the structure (that could allow the restoring of their redistribution). This molecule reached Phase II trials.[4]

Mono-8-hydroxyquinoline entities such as clioquinol form, after deprotonation of the phenolic function, stable metal complexes in 1/1 (LM) or 2/1 (L_2M) ligand/metal ratios.[5] To evaluate the capacity of tetradentate ligands to directly form L_2M species compared to mono-hydroxyquinoline ligands (bidentate ligand) as potential anti-Alzheimer chelating agents, we report here the properties of ligand **1**, containing two 8-hydroxyquinoline entities covalently linked together (Scheme 1).[6] This tetradentate ligand allows, from molecular models, the formation of Cu(II) or Zn(II) complexes, with both quinolines chelating the metal ion (Fig. S1†). The linker between the two quinoline entities is at the C2 position of each heterocycle, with a short methylene bridge to favor mononuclear metal complexes. **1** is also symmetric and without chiral centers for synthetic purposes in drug development. Ligand **1** should be considered as a prototype for studies related to tetradentate ligands as potential therapeutic agents.

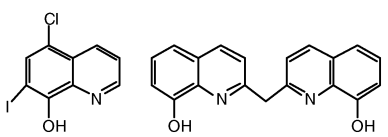
Due to the easy formation of stable metal complexes with **1** compared to the necessity of recruiting two mono 8-hydroxyquinoline entities, especially in a living organism, an increased ability of such tetradentate ligands to efficiently remove metal ions from amyloid aggregates can be expected. The possibility of modulating the metal ion content of amyloid structures is targeted. Ligand **1**, and also its Cu(II) and Zn(II) complexes, are small compounds (MW < 500), neutral at physiological pH and hydrophobic; three required properties that facilitate the crossing of the blood–brain barrier (BBB) from blood-to-brain and from brain-to-blood.[7] Importantly, when different strong chelators were tested *in vitro* to dissolve amyloid plaques or aggregates, they were generally hydrophilic and unable to cross the BBB, a parameter that limited the study of their potential activity *in vivo*.[3e,f,8] Therefore, the development of chelators such **1** could be attractive to obtain new tools to study the role of metal ions in AD. Importantly also, hydrophobic chelators such as XH1[3c] or DP-109[3d] had positive effects on transgenic mice, mimicking AD without apparent toxicity.

The ability of **1** to chelate Zn(II), Cu(II) and Fe(III), three metal ions involved in Aβ aggregation, was analyzed by mass spectrometry. After incubation with 1 equiv. of metal ion, complex formation was confirmed by chemical ionization, and $[MH]^+$ complexes were observed at m/z = 381, 382 and 397 for **1** · Cu(II), **1** · Zn(II) and **1** · Fe(III)Cl, respectively.

The partition of **1** between octanol and a physiological aqueous phase was measured, and the log $D_{7.4}$ value was 3.3 (compared to 3.8 for clioquinol, known to cross the BBB).

The ability of **1** (40 equiv.) to inhibit the precipitation of $A\beta_{1-42}$ (1 equiv.) in the absence or presence of Cu(II), Zn(II) or Fe(III) (4 equiv.) was compared to clioquinol (Table 1) under conditions similar to those used by Atwood *et al.*[8]

Without the addition of metal ion, 45% of $A\beta_{1-42}$ was precipitated. The addition of both ligands decreased the aggregation of $A\beta_{1-42}$ in insoluble forms (this effect can also



Clioquinol **1**

Scheme 1 The compounds studied here.

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Table 1 Percentage of soluble $A\beta_{1-42}$[a]

	No ligand	**1**	Clioquinol
No metal	55 ± 4	63 ± 4	63
$CuCl_2$ 4 equiv.	18 ± 2	60 ± 5	55 ± 3
$CuCl_2$ 2.5 equiv.	20 ± 4	59 ± 4[b]	35 ± 3[c]
$ZnCl_2$ 4 equiv.	29 ± 3	63	37
$FeCl_3$ 4 equiv.	47 ± 2	61	51

[a] $A\beta_{1-42}$ (5 μM) was incubated for 1 h at 37 °C in 20 mM tris·HCl buffer (pH 7.4), 150 mM NaCl with or without 20 or 12.5 μM metal salt ($CuCl_2$, $ZnCl_2$ or $FeCl_3$), then for another 1 h in the presence of 200 μM ligand, followed by centrifugation. Quantities of soluble and precipitated $A\beta_{1-42}$ were determined in the supernatant and pellet, respectively, with MicroBCA assay. [b] For 12.5 μM of **1**. [c] For 25 μM of clioquinol.

be due to the chelation of residual traces of metal ions inducing the peptide's precipitation) but the major effects were, however, observed when $A\beta_{1-42}$ was aggregated with metal ions.

In the presence of Cu(II), only 18% of $A\beta_{1-42}$ remained soluble under the experimental conditions used. Controls showed that maximal peptide precipitation was obtained after 1 h of incubation and did not change after 2 h. Addition of both ligands increased the solubility of $A\beta_{1-42}$. Interestingly, the use of **1** allowed the restoration of 100% of the value of soluble $A\beta_{1-42}$ observed in the absence of added metal ion (63% of soluble $A\beta_{1-42}$ in the presence of this ligand). From the same type of analysis, clioquinol was less efficient, since only 80% of the maximum possible solubility was observed.

In the presence of Zn(II), 29% of $A\beta_{1-42}$ peptide remained soluble. Comparison with the same experiment performed in the presence of Cu(II) (18% of soluble $A\beta_{1-42}$) shows that the method used reflects the minor ability of Zn(II) to aggregate the peptide. The addition of **1** to $A\beta_{1-42}$ pre-incubated with Zn(II) again allowed 100% of the solubility observed in the absence of added metal ion to be obtained, while clioquinol was poorly efficient under these conditions.

The addition of Fe(III) induced only a minor increase of $A\beta_{1-42}$ precipitation compared to the level in the absence of added metal ion. Also in this case, **1** is efficient since ~100% of the solubility observed in the absence of added metal was restored. The experiment was inconclusive in the case of clioquinol.

Previous experiments were performed with an excess of metal ion and ligand, and small differences were observed between both ligands when $A\beta_{1-42}$ was pre-incubated with Cu(II), which has a most dramatic effect on the precipitation of $A\beta_{1-42}$. The Cu(II)/peptide ratio that induced the maximum aggregation of insoluble forms was determined (Fig. S2†). Precipitation increased when the Cu(II)/$A\beta_{1-42}$ ratio increased until a ratio of 2.5 Cu(II) per $A\beta_{1-42}$, then it stabilized at 82% peptide. This value was in accordance with the literature proposition that $A\beta_{1-42}$ can complex 2.5 Cu(II) ions.[8a] To refine the results obtained with the ligands, the "highest" aggregating conditions with a minimum of 2.5 equiv. of Cu(II) per peptide were tested. In this new series of experiments, we aimed to analyze the solubility without an excess of metal ion or chelator. Therefore, $A\beta_{1-42}$ peptide (5 μM) was incubated for 1 h with 2.5 equiv. of Cu(II) and for 1 h in the presence of 2.5 equiv. of **1**, having the metal/ligand ratio 1/1. For clioquinol, 5 equiv. of ligand were added, since complexes with a 2/1 ligand to Cu(II) ratio have been classically reported.[5] A significant decrease in the ability of clioquinol to inhibit $A\beta_{1-42}$ precipitation was observed when experiments were performed without an excess of Cu(II) and ligand, reflecting the necessity of an excess of clioquinol for efficiency against peptide precipitation due to Cu(II). More importantly, in the case of **1**, is the fact that a 1/1 ratio of Cu(II)/ligand was sufficient to obtain the maximum effect and no excess of ligand was necessary. These results could reasonably be correlated to a better affinity of the bis-8-hydroxyquinoline ligand for Cu(II) compared to the mono-8-hydroxyquinoline clioquinol.

Aβ–Cu in the presence of a reductant exerts a toxicity correlated with the generation of H_2O_2, and inhibitors of this catalytic activity might be of therapeutic value.[2] The ability of the chelators to inhibit the production of H_2O_2 by $CuCl_2$ in the absence of $A\beta_{1-42}$, or for Cu/$A\beta_{1-42}$ ratios of 2/1 and 0.75/1, was compared (Table 2). Under the conditions used, $A\beta_{1-42}$, $CuCl_2$ or copper complexes of $A\beta_{1-42}$ did not generate a significant quantity of H_2O_2 in the absence of ascorbate. However, copper complexes ($A\beta_{1-42}\cdot Cu_n$ or $CuCl_2$) in the presence of ascorbate were catalytically able to produce H_2O_2.[9]

In the absence of $A\beta_{1-42}$, both ligands inhibited H_2O_2 production mediated by $CuCl_2$ in the presence of ascorbate (2.93 nmol of H_2O_2). Clioquinol was less active than **1** against the $CuCl_2$-mediated reaction since 2 equiv. of clioquinol were necessary to decrease significantly the H_2O_2 production of 82% when only one equiv. of **1** was sufficient to obtain a similar effect.

Both chelators were able to inhibit H_2O_2 production mediated by $A\beta_{1-42}\cdot Cu_n$ complexes in the presence of ascorbate. For a Cu/$A\beta_{1-42}$ ratio = 2, both ligands, in the proportion one equiv. per copper ion, reduced the production of H_2O_2 to 25–30% of the quantity produced by $A\beta_{1-42}\cdot Cu_2$ in the absence of chelator (only 0.75–0.89 nmol of H_2O_2 were produced compared to 2.77 nmol without added ligand). For a Cu/$A\beta_{1-42}$ ratio = 0.75, the copper complex of the amyloid peptide produced less H_2O_2 (1.08 nmol), and again one equivalent of both chelators per copper inhibited significantly the production of H_2O_2.

In conclusion, a strategy based on the use of a tetradentate ligand containing two linked 8-hydroxyquinolines allowed significant increases in the ability to protect against β-amyloid peptide precipitation in the presence of Cu(II), Zn(II) and Fe(III) compared to clioquinol, a 8-hydroxyquinoline

Table 2 Inhibition of the production of H_2O_2[a]

	No $A\beta_{1-42}$	Cu/$A\beta_{1-42}$ = 2	Cu/$A\beta_{1-42}$ = 0.75
No ligand	2.93 ± 0.18	2.77 ± 0.16	1.08
1 1 equiv.	0.54	0.75 ± 0.11	0.76
Clioquinol 1 equiv.	2.07	0.89 ± 0.08	0.64
Clioquinol 2 equiv.	0.54	0.78 ± 0.06	0.57

[a] Experiments were performed in the presence of 0.4 μM $CuCl_2$, 10 μM ascorbate and air. Generated H_2O_2 (in nmol) was quantified with AmplexRed assay.

derivative active *in vivo*. The bis-8-hydroxyquinoline ligand can also inhibit the production of H_2O_2 induced by Cu–Aβ_{1-42}, associated with toxic oxidative stress in AD. Hydrophobic chelator **1** is active in a 1/1 ratio with the metal ion. These preliminary results validate prerequisites for the design of these ligands, which now have to be tested with more biologically significant experiments before considering **1** as a good prototype in selective chelato-therapy. Although metal chelation may be a promising therapeutic strategy for AD, a major problem associated with the widespread clinical use of metal complexing agents could be poor target specificity and consequently limited clinical safety. Answering these questions is also related to the development, by biologists, of efficient cellular and animal models to facilitate the screening of drug candidates in the field of neurodegenerative diseases.

Experimental

Analysis of Aβ_{1-42} precipitation according to the peptide/Cu(II) ratio (final concentrations are given)

500 μL of Aβ_{1-42} (5.0 μM) was induced to aggregate by incubation in 20 mM tris·HCl/150 mM NaCl buffer (pH 7.4) with different ratios of $CuCl_2$ in DMSO (50 μL) for 2 h at 37 °C under stirring at 1400 rpm. Samples were then centrifuged for 20 min at 5500 g and 500 μL of supernatant was removed. The test tube containing residual supernatant and pellets (corresponding to the precipitated Aβ_{1-42}) received 450 μL of the experimental buffer/DMSO (91/9, v/v). Then, both supernatant and pellets were analyzed for the determination of protein concentration by MicroBCA assay. This double quantification allowed the initial quantity of peptide to be monitored and so confirm the validity of the measurements.

Log $D_{7.4}$ determination,[10] inhibition of Aβ_{1-42} precipitation[8] and hydrogen peroxide assay[9] were adapted from literature (see ESI for detailed procedures and general remarks†).

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