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Effects of Clioquinol on Metal-Triggered Amyloid-β Aggregation Revisited

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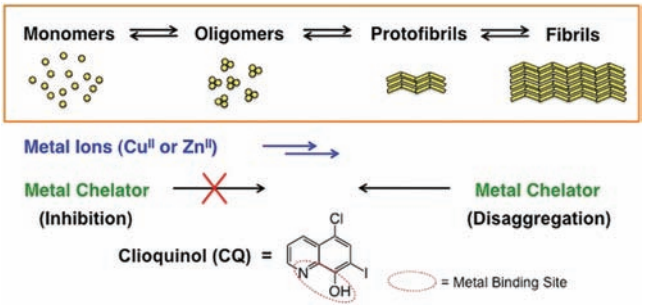
Amyloid-β (Aβ) plaques are largely associated with the neuropathogenesis of Alzheimer's disease (AD). Metal ions such as Cu^{II} and Zn^{II} have been implicated as contributors to their formation and deposition. Metal chelators have been used to modulate metal-induced Aβ aggregation. The bidentate ligand clioquinol (CQ) presents an effective influence on metal-involved Aβ aggregation, which has been explained through its metal chelation and is generally monitored by fluorescence and turbidity assays in vitro. The studies herein, however, suggest that the effects of CQ on metal-driven Aβ aggregation may not be visualized accurately by both assays. Subsequently, the present work demonstrates that CQ is able to chelate metal ions from metal−Aβ species and to assist, in part, in the disaggregation of Aβ aggregates, but it could not completely hinder the progression of Aβ aggregation.

Alzheimer's disease (AD), a currently incurable, progressive neurodegenerative disorder, is the leading cause of dementia in the elderly, affecting nearly 11 million people worldwide.[1−3] Memory loss and cognitive decline are distinctive symptoms of the disease and are potentially severe enough to be terminal within a decade after diagnosis. One hallmark of AD is the presence of accumulated amyloid-β (Aβ) plaques. Causes leading to the development of Aβ deposits are not well understood.[3−5] It has been suggested that metal ions such as Cu^{II} and Zn^{II} are involved in the assembly and neurotoxicity of Aβ species.[1,3−6] Metal ions are able to accelerate the formation of Aβ aggregates and influence their conformational transformation. In addition, the formation of reactive oxygen species (ROS) by Cu−Aβ has been suggested as one proposed mechanism of AD pathogenesis.[3,5−10] The Aβ-bound Cu^{II} (Cu^{II}−Aβ) may catalytically generate hydrogen peroxide in the presence of dioxygen and reducing agents. This could cause an increase in oxidative stress, which triggers damage of cellular components such as DNA, lipids, and proteins.[3,9,10]

Scheme 1. Proposed Influence of CQ on the Progression of Metal-Induced Aβ Aggregation



As logically expected, metal-ion chelation therapy as a treatment for AD has been explored to prevent Aβ deposition and ROS production.[5,11−18] The generation and resolubilization of metal-associated Aβ aggregates could be controlled by metal chelators (Scheme 1). Among several chelators, clioquinol (CQ), known as an antiamoebic

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compound, significantly reduces Aβ plaque deposits, leading to improved cognitive behavior, in early phase II clinical trials.[5,18] Unfortunately, the long-term use of CQ is limited by an adverse side effect, subacute myelo-optic neuropathy.[19,20] Even though CQ is not currently used as a therapeutic agent in AD treatment, its in vivo studies demonstrate that metal-ion chelation therapy is a promising route for curing AD.

The mechanism behind the effectiveness of CQ shown in vitro and in vivo is still elusive. Structural characterization of the metal(CQ) complexes (metal = Zn^{II} or Cu^{II}) reveals a 1:2 metal-to-ligand ratio showing a trigonal-bipyramidal geometry for $[Zn(CQ)_2(H_2O)]$ and square-planar geometry for $[Cu(CQ)_2]$ (CQ is deprotonated in both structures).[21] The influence of CQ on the inhibition of the formation of metal-induced Aβ species as well as their disaggregation in vitro (Scheme 1) has been monitored by fluorescence [Thioflavin-T (ThT)] and turbidity assays.[11,12,17] Both analytical methods, however, may not be suitable for determining the degree of Aβ aggregation in samples containing metal ions, CQ, and Aβ. The general windows for both ThT (λ_{ex} = ca. 440 nm; λ_{em} = 490 nm) and turbidity (ca. 350−450 nm) measurements can overlap with the absorption of CQ and its corresponding metal complexes.[11−17,22−24] Consequently, it is important to reevaluate the ability of CQ to chelate the metal ions from metal-associated Aβ species and to control the progression of Aβ aggregation. Here we report visualization of how CQ modulates the generation and disaggregation of metal-induced Aβ species by transmission electron microscopy (TEM), BCA protein analysis, and circular dichroism (CD) spectroscopy. These studies demonstrate that metal chelation by CQ occurs and CQ aids in the partial disassembly of Aβ aggregates, but it could not completely prohibit Aβ aggregation.

To study the impact of CQ on metal-induced Aβ species, two separate experiments were performed: its influence on the formation of metal-triggered Aβ aggregates (inhibition experiments; Figure 1, top) and its effect on the disassembly of metal fibrils (disaggregation experiment; Figure 2, top). The degree of Aβ aggregation was verified by TEM, BCA protein analysis, and CD.[25] All samples containing Cu^{II} and Zn^{II} were buffered at pH 6.6 and 7.4, respectively.[13a]

For inhibition experiments (Figure 1), the samples were prepared as follows: freshly prepared Aβ was first treated with metal ions for 2 min and introduced to CQ for 24 h at 37 °C with constant agitation. The TEM images illustrate significant Aβ aggregation from the samples of the inhibition experiments (Figure 1, bottom). Upon incubation of Aβ with

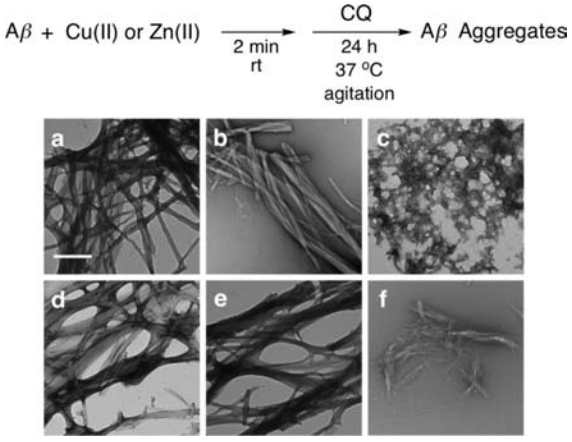

Figure 1. Scheme of inhibition experiments (top). TEM images (bottom) of samples containing (a) metal-free Aβ (pH 6.6), (b) Aβ and Cu^{II}, (c) Aβ, Cu^{II}, and CQ, (d) metal-free Aβ (pH 7.4), (e) Aβ and Zn^{II}, and (f) Aβ, Zn^{II}, and CQ ([Aβ] = 25 μM, [metal] = 25 μM, and [CQ] = 50 μM). Samples were incubated for 24 h at 37 °C with constant agitation. The scale bar depicted in the figure indicates 500 nm.

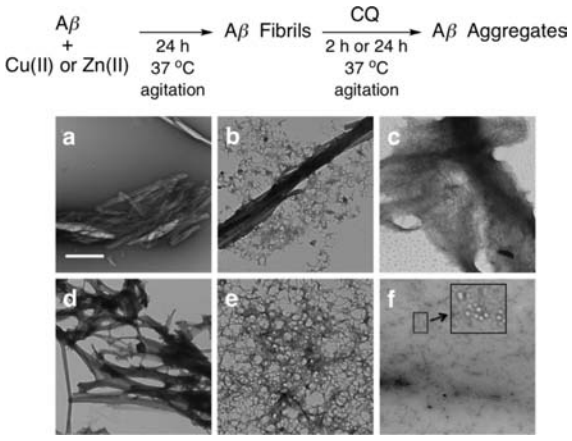

Figure 2. Scheme of disaggregation experiments (top). TEM images (bottom) of (a) Cu^{II}−Aβ fibrils at pH 6.6, Cu^{II}−Aβ fibrils incubated with CQ (b) for 2 h and (c) for 24 h, (d) Zn^{II}−Aβ fibrils (pH 7.4), Zn^{II}−Aβ fibrils incubated with CQ (e) for 2 h and (f) for 24 h (inset: expanded area showing spherical species) ([Aβ] = 25 μM, [metal] = 25 μM, [CQ] = 50 μM, 37 °C, constant agitation, scale bar = 500 nm).

or without metal ions, well-ordered, mature fibrils are observed (Figure 1a,b,d,e). On the other hand, when CQ is added into the samples of Aβ and metal ions, clusters of short fibrils with mature fibrils are present (Figure 1c,f). In addition, samples of metal-free Aβ treated with CQ or the Cu(CQ) complex, generated in situ by reacting CQ with Cu^{II} in a ratio of 2:1, also show Aβ aggregates containing a mixture of defined and amorphous Aβ species (Figure S2 in the Supporting Information). The morphology of these Aβ species shown in Figure S2 is different from that of CQ-treated metal−Aβ (Figure 1c,f). Taken together, these TEM results suggest that CQ is capable of triggering the different organization of Aβ aggregates, but Aβ aggregation could still be progressing following its metal chelation.

The contents of soluble Aβ species in the inhibition experiments were also determined by BCA protein analysis. Over a 24-h period of incubation time, a significant decrease of soluble Aβ (%) in metal-containing Aβ samples (< 10%) over metal-free Aβ samples (ca. 70%) occurs (Figure S3 in the Supporting Information). The metal−Aβ samples treated

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(25) The predominant Aβ species were depicted in the TEM figures (Figures 1 and 2 and Figures S2 and S4 in the Supporting Information).

with CQ show no distinguishable difference in the amount of soluble Aβ, compared to that from samples without CQ at early time frames (< 1 h), suggesting that CQ may not inhibit initial metal-induced Aβ aggregation. On the other hand, the amount of soluble Aβ is increased to ca. 30% in the CQ-treated samples of metal−Aβ over the duration of incubation from 1 to 24 h, while it is further reduced to < 10% from the metal−Aβ samples.[26] This could have resulted from the limited inhibition of the generation of Aβ aggregates, their partial disaggregation, or both by CQ over a period of incubation (infra vide). Overall, the analyses of the inhibition samples by TEM and BCA reveal that CQ may hinder slightly the progression of Aβ aggregation, provoking structural alteration of Aβ aggregates.

For the disaggregation studies (Figure 2), CQ was treated for 2 or 24 h with Aβ fibrils, generated by reacting freshly prepared Aβ with or without metal ions for 24 h at 37 °C with continuous agitation. Upon treatment of CQ with well-defined, mature metal−Aβ fibrils (Figure 2a,d), their significant structural transformation is visible by TEM (Figure 2, bottom). In the Cu^{II} sample, the morphology of Cu^{II}−Aβ fibrils is altered from mature, structured fibrils to a mixture of short aggregates and defined fibrils (after 2 h, Figure 2b) or to hairlike fibrils (after 24 h, Figure 2c). More markedly, the mature, well-ordered Zn^{II}−Aβ fibrils are transformed into a combination of short fibrils and a small portion of spherical species[23] after 24 h incubation (Figure 2f). In addition, metal-free Aβ fibrils with CQ are also reshaped to short and/or long, undefined fibrils but not to spherical species (Figure S4 in the Supporting Information). These observations suggest that CQ induces the disassembly of Aβ fibrils, but the complete disaggregation of Aβ aggregates by CQ does not take place.

The percentage of soluble Aβ from samples of the disaggregation experiments is increased upon the addition of CQ to Cu^{II}− and Zn^{II}−Aβ fibrils, relative to that from samples of CQ-untreated metal−Aβ fibrils (Figure S5 in the Supporting Information; from ca. 9% to 20% for the Cu^{II} samples and from ca. 4% to 14% for the Zn^{II} samples). This enhanced content of soluble Aβ could be a consequence of the disaggregation of metal−Aβ fibrils by CQ, as shown in the TEM images of Figure 2. Still, the overall amount of soluble Aβ from the CQ-treated samples of metal−Aβ is less than that from samples of metal-free Aβ incubated with CQ. In addition, the Zn^{II}-triggered β-sheet structure of Aβ, which can be monitored by CD,[14,16] is very slightly reduced after 5 h incubation with CQ (Figure S6 in the Supporting Information). The β-sheet conformation of metal-induced Aβ fibrils even after 24 h incubation of CQ is monitored by CD. Thus, the results of the disaggregation experiments suggest that CQ could refashion the structural conformation of Aβ fibrils, but recovery of small, soluble Aβ species is limited through the disassembly of well-structured metal−Aβ fibrils by CQ.

On the basis of the observations from the experiments above, treatment of CQ does not completely hinder Aβ aggregation and break down Aβ fibrils. From this, a question arises, does metal chelation occur when CQ is allowed to react with metal−Aβ species? Immediately after the addition of CQ to the Cu^{II}−Aβ samples in both experiments of inhibition and disaggregation, the optical band at 450 nm is clearly evident (Figure S7 in the Supporting Information). This band corresponds to a ligand−metal charge transfer from the Cu(CQ) complex. Thus, CQ is able to capture metal ions from metal-associated Aβ species,[11a] but this metal chelation could not fully prevent the development of Aβ aggregation.

The bidentate ligand CQ has been known as an inhibitor and/or resolubilizer of metal-triggered Aβ aggregates upon its metal coordination. The effects of CQ on Aβ aggregation have been visualized by the ThT and turbidity assays generally employed. The present work, however, suggests that these methods may not be suitable for determining the degree of metal-assisted or metal-free Aβ aggregation in the presence of CQ because the windows of analyses interfere with the absorptions of CQ and its corresponding metal complexes. Following this, our reinvestigation on the influence of CQ on Aβ aggregation using TEM, BCA protein analysis, CD, and UV−vis demonstrate that it chelates metal ions from metal−Aβ species and causes conformational transformation of Aβ aggregates. The compound CQ, however, assists in the partial disaggregation of metal-induced Aβ aggregates and limited inhibition of their formation in vitro. Still, the significant effects of CQ on the modulation of neurotoxic Aβ species and the reduction of Aβ plaque deposits in vivo, reported previously,[5,11,12,18] remain unclear. One possibility is that metal chelation by CQ may generate a less toxic species of Aβ and could be efficient at regulating metal-ion homeostasis, consequently activating matrix metalloproteases that would degrade Aβ.[5,11,27]

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Supporting Information Available: Experimental procedures and Figures S1−S7. This material is available free of charge via the Internet at http://pubs.acs.org.

(26) After 24 h of incubation, less soluble Aβ species are still indicated in the CQ-treated samples of metal−Aβ (~30%) over those of the metal-free Aβ (60−70%).

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