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Effects of Cyclen and Cyclam on Zinc(II)- and Copper(II)-Induced Amyloid β-Peptide Aggregation and Neurotoxicity

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The aggregation of amyloid β-peptide (Aβ) in plaques in brain tissue is highly associated with Alzheimer's disease (AD). Aberrant homeostasis of cerebral metals such as Zn^{2+} and Cu^{2+} may facilitate the formation of the pathogenetic amyloid plaques. Further, the accumulation of redox-active Cu^{2+} in these plaques leads to the generation of reactive oxygen species, which mediates the conspicuous oxidative damage to the brain in AD. In this study, the effect of macrocyclic polyamine chelators, cyclen and cyclam, on the aggregation of Aβ40 induced by Zn^{2+} or Cu^{2+} was investigated using turbidometry, thioflavin T fluorescence spectroscopy, electrospray ionization mass spectrometry, inductively coupled plasma mass spectrometry, BCA protein assay, circular dichroism spectroscopy, and atomic force microscopy. The solubility of Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregates is greatly increased by cyclen or cyclam as compared to that without chelators, and the solubilization is not affected by other essential metal ions such as Ca^{2+} and Mg^{2+}. Moreover, the metal-induced β-sheet structure of Aβ40 can be reconverted to its original random coil conformation, and the generation of H_2O_2 mediated by the Cu-Aβ40 complex can also be inhibited by these chelators. Preliminary tests on neuronal cells indicate that these chelators are capable of reducing the toxicity of metal-Aβ40 aggregates. These observations suggest that cyclen and cyclam could be lead compounds as neuroprotective or neurorescue agents for the treatment of AD.

Introduction

Alzheimer's disease (AD) is a neurodegenerative malady characterized by the aggregation of amyloid β-peptide (Aβ) in plaques in brain tissue.[1−3] Aβ40 and Aβ42 are the most abundant Aβ forms, with the former being the major species.[4,5] Aβ possesses selective high- and low-affinity binding sites for Zn^{2+} and Cu^{2+} that are mainly mediated by histidine residues, and the binding to these ions makes Aβ deposit.[6,7] Even a trace amount of Zn^{2+} or Cu^{2+} is sufficient to induce the nucleation of Aβ, and Zn^{2+} is particularly powerful in inducing such aggregations.[8−10] The binding ratio of Zn^{2+} and Cu^{2+} to Aβ varies from 1 to 2.5 at physiological pH.[11] Many studies suggest that aberrant homeostasis of cerebral Zn^{2+} and Cu^{2+} may contribute to the AD pathogenesis by facilitating the formation of amyloid deposits and enhancing the generation of reactive oxygen species and toxic Aβ oligomers in the brain.[12−14] In fact, the concentrations of Zn^{2+} and Cu^{2+} are markedly elevated in cerebral Aβ deposits of AD patients.[15,16] Experimental and clinical data clearly indicate that interference in Aβ-metal interactions may reverse the neurotoxic effect of

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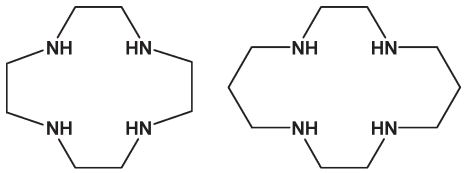
Aβ aggregates and provide promising therapeutic modality for AD treatment.[17]

Chelators of Zn^{2+} and Cu^{2+} are proposed as potential therapeutic agents for AD because Zn^{2+}- or Cu^{2+}-induced Aβ aggregation is completely reversible toward chelation,[18,19] and the adverse redox reactions of the metal-Aβ aggregates can be inhibited by chelators.[20] Several chelators have been shown to greatly enhance the dissolution of insoluble Aβ aggregates in both in vitro and in vivo trials.[21,22] For example, clioquinol (CQ), a bioavailable hydrophobic Zn^{2+} and Cu^{2+} chelator, was shown to significantly reverse Zn^{2+}- or Cu^{2+}-mediated aggregation of Aβ in vitro and inhibit Aβ accumulation in AD transgenic mice,[21] and a phase II clinical trial on the effects of oral CQ in AD patients has been completed. Nevertheless, the overdose-associated neurological side effects of CQ still remain to be overcome. Chelators such as *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylene diamine, bathocuproine, trientine, penicillamine, and bathophenanthroline are also effective in solubilizing brain Aβ deposits.[23,24] However, these chelators are too hydrophilic to cross the blood−brain barrier (BBB), and consequently their use in vivo is rather dim. BBB-permeable lipophilic chelators such as DP-109, XH1, and Cyclo-Phen-type molecules were thus designed and shown to remarkably reduce Zn^{2+}- or Cu^{2+}-induced Aβ aggregates in vivo in mice models.[25−27] More recently, derivatives of a 14-membered tetramine, 1,1′-xylyl bis-1,4,8,11-tetraazacyclotetradecane, have been shown to be effective in reducing the copper concentration in the brain cortex and to be able to maintain normal copper levels in the blood, cerebro spinal fluid, or corpus callossum of rats.[28]

Macrocyclic chelators 1,4,7,10-tetraazacyclododecan (cyclen) and 1,4,8,11-tetrazacyclotetradecane (cyclam) are important polyamines because metal complexes of their derivatives have many applications in medicine.[29,30] The toxicity of cyclen and cyclam is very low, with the LD_{50} value being 387.6 and 370.6 mg/kg,[31,32] respectively. They exhibit considerable affinity for both Zn^{2+} and Cu^{2+} ions, but the stability constant of their Zn complexes (log $K_{\text{Zn-cyclen}}$ = 16.2, log $K_{\text{Zn-cyclam}}$ = 15.5)[33] is much less than that of Cu complexes (log $K_{\text{Cu-cyclen}}$ = 24.8, log $K_{\text{Cu-cyclam}}$ = 28.1).[34,35] Accordingly, the coordination with the Cu^{2+} ion is more favorable for these chelators, and the biologically less active Cu-cyclam complex is more likely to be formed in vivo.[36] Here, we report the effects of cyclen and cyclam on the Aβ peptide aggregation induced by Zn^{2+} or Cu^{2+} and on the toxicity correlated with the generation of H_2O_2 induced by Cu^{2+}.



cyclen cyclam

Experimental Section

Materials and Measurements. Human Aβ40 was synthesized by solid-phase Fmoc chemistry at GL Biochem Ltd. (Shanghai, China) and was purified after removal from the resin and deprotection by reverse-phase high-performance liquid chromatography with UV−vis detection. The final sample was verified by electrospray ionization mass spectrometry (ESI-MS). Zinc acetate dehydrate, copper chloride, thioflavin T (ThT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and tris(hydromethyl)aminomethane (Tris) were purchased from Sigma-Aldrich. Other chemicals were of analytical grade. A peptide stock solution was prepared by dissolving Aβ40 (2.0 mg) in NaOH (500 μL, 20 mM) and sonicating it for 1 min in a water bath to avoid frothing. This solution was diluted with water (500 μL) and sonicated for 1 min and adjusted to pH 7.4 using NaOH (0.5 M) and HCl (0.5 M) and then filtered through a 0.22 μm filter (Millipore). The concentration of Aβ40 was immediately determined with a BCA Protein Assay Kit (Pierce) using bovine serum albumin as a standard. The stock solution was kept in a refrigerator for later use. Stock solutions of cyclen and cyclam were prepared by dissolving the respective chelator in water to get a final concentration of 4 mM, and those of $Zn(Ac)_2$ and $CuCl_2$ were prepared by dissolving the respective salt in water to reach a concentration of 5 mM. All solutions and buffers used in this study were prepared with deionized water and filtered (pore size 0.22 μm, Millipore) before use to remove any granular substance. The Aβ40-related experiments were performed at least in triplicate with the discrepancy between individual values less than 5%. All of the resolving processes in this study lasted 4 h to ensure that the system reached a steady state.

Determination of Turbidity. The sample solution (100 μL) consisting of Aβ40 (50 μL, 200 μM), $Zn(Ac)_2$ or $CuCl_2$ (4 μL, 5 mM), and a buffer (10 μL, 200 mM Tris-HCl/1.5 M NaCl, pH 7.4) was incubated at 37 °C for 2 days. Cyclen or cyclam (10 μL, 4 mM) was added to the solution followed by incubation

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at 37 °C for 4 h. The sample was then mixed with a buffer (900 μL, 20 mM Tris-HCl/150 mM NaCl, pH 7.4) and examined on a Lambda 35 UV−vis spectrometer (Perkin-Elmer) using a cuvette (0.5 cm). Turbidity of the sample solution was evaluated indirectly by measuring the UV−vis absorbance at 405 nm. Solutions containing the same amount of Aβ40, Zn^{2+}, or Cu^{2+} alone were used as controls.

Measurement of Fluorescence. The sample solutions were prepared in the same way as described above. Each sample (100 μL) was transferred to a cuvette (1 cm) containing ThT (900 μL, 13.3 μM) dissolved in a buffer (20 mM Tris-HCl/150 mM NaCl, pH 7.4) and shaken for 30 s in the dark and incubated for 5 min before testing. Fluorescence spectra in the range of 440−650 nm (λ_{ex} = 415 nm) were recorded on an AMINCO Bowman Series 2 Luminescence spectrometer, and an average of three scans was taken as the final result.

Monitoring of Peptide. Sample solutions prepared as above were centrifuged at 12 000 rpm for 30 min, and the peptide concentration in the supernatant was analyzed with a BCA Protein Assay Kit. Samples for the ESI-MS experiment were prepared as follows: Before addition of the chelator, each of the aforesaid sample solutions was centrifuged (12 000 rpm, 30 min). The precipitate was washed with water and centrifuged again (12 000 rpm, 30 min), and the precipitate was resuspended in water (100 μL). Cyclen or cyclam (5 μL, 4 mM) was added to the solution and incubated at 37 °C for 4 h. The pH value of the solution was adjusted to 7.4 with NaOH (0.5 M) and HCl (0.5 M). The solution was centrifuged (12 000 rpm, 30 min), and the supernatant was subjected to analysis using ESI-MS. The ESI-MS spectra were recorded on an LCQ electron spray mass spectrometer (Finnigan).

Detection of Metal Ions. Sample solutions described in the turbidimetry experiment were centrifuged (12 000 rpm, 30 min). The precipitate was washed with a buffer (20 mM Tris-HCl/150 mM NaCl, pH 7.4) and centrifuged again (12 000 rpm, 30 min), and the precipitate was resuspended in the above buffer (100 μL). Cyclen or cyclam (10 μL, 4 mM) or water (10 μL) was added to the solution, and the mixture was incubated at 37 °C for 4 h. The supernatant (80 μL) was taken out after centrifugation (12 000 rpm, 30 min) and diluted with HNO_3 (2%, 3 mL) for analysis. The content of the metal in the Zn- or Cu-Aβ40 sample was determined by inductively coupled plasma mass spectrometry (ICP-MS) on an Elan 900 instrument (Perkin-Elmer, USA) in the peaking-hoping mode. The apparatus was calibrated using a HNO_3 (2%) solution containing Cu^{2+} and Zn^{2+} (5, 10, 50, and 100 ppb), with In115 being the internal standard for all isotopes of Cu^{2+} and Zn^{2+}. The average value of three parallel tests was taken as the final result.

Investigation of Conformation. Mixed solutions (500 μL) containing Aβ40 (125 μL, 200 μM), $Zn(Ac)_2$ or $CuCl_2$ (10 μL, 5 mM), and a phosphate buffer (50 μL, 100 mM, pH 7.4) were kept at 37 °C for 1 day. Cyclen or cyclam (25 μL, 4 mM) was added to each solution and then incubated at 37 °C for 4 h. Circular dichroism (CD) spectra of the samples in the range of 190−260 nm were recorded on a JASCO J-810 automatic recording spectropolarimeter (Tokyo, Japan) controlled by the Jasco software. The data of the baseline acquired in the absence of a peptide were subtracted from each spectrum.

Protective Capability of the Chelators. $Zn(Ac)_2$ or $CuCl_2$ (2 μL, 5 mM) was added to the solution (98 μL) containing Aβ40 (50 μL, 200 μM), cyclen or cyclam (2.5 or 5 μL, 4 mM), and a buffer (10 μL, 200 mM Tris-HCl/1.5 M NaCl, pH 7.4). The mixture was incubated at 37 °C for 4 h, and the amount of soluble Aβ40 was determined with a BCA Protein Assay Kit after centrifugation.

Selectivity of the Chelators. A sample solution (100 μL) containing Aβ40 (50 μL, 200 μM), $Zn(Ac)_2$ (2 μL, 5 mM), $CuCl_2$ (2 μL, 5 mM), and a buffer (10 μL, 200 mM Tris-HCl/1.5 M NaCl, pH 7.4) was kept at 37 °C for 2 days. $CaCl_2$ (4 μL, 50 mM), $MgCl_2$ (4 μL, 50 mM), and water (10 μL) or cyclen (10 μL, 4 mM) or cyclam (10 μL, 4 mM) was added to the solution. The mixture was incubated at 37 °C for 4 h, and the content of soluble Aβ40 was determined with a BCA Protein Assay Kit after centrifugation.

Atomic Force Microscopy. Aβ40 sample solutions for atomic force microscopy (AFM) were prepared as described in the above conformation test and used after sufficient vortical mixing. Aliquots (5 μL) of each Aβ40 sample were deposited onto freshly cleaved mica (Φ 1 cm) and incubated at room temperature for 3 min. The remaining salts and loose deposits in suspension were subsequently rinsed with ultrapure water (50 μL, Millipore) and then dried with N_2. AFM images were obtained on a Nanoscope V Multimode scanning probe workstation using etched silicon Nano-Probes (probe model AC160TS, Digital Instruments, Olympus) under ambient conditions. AFM analysis was carried out in a tapping mode, and images were acquired at a resolution of 512 × 512 pixels. Typical scanning parameters were as follows: initial root-mean-square amplitude, 0.33 V; set point, 400−500 mV; tapping frequency, 300−320 kHz; scan rate, 2 Hz. Nanoscope VII software provided by the manufacturer of the AFM instrument was used to measure the volume distributions (height, width, and length) of Aβ40 deposits on mica. At least five spots in the entire area were analyzed in each case.

Testing of H_2O_2. DCFH-DA (10 mM) was deacetylated by NaOH (0.05 M) in argon-purged O_2-free dimethyl sulfoxide for 30 min. The system was neutralized to pH 7.4, and the final concentration of DCFH was diluted to 1 mM with a buffer (20 mM Tris-HCl/150 mM NaCl, pH 7.4). The reactions among DCFH (100 μM) and freshly prepared Aβ40 (300 nM), $CuCl_2$ (150 nM), and ascorbate (2 μM) were carried out in a buffer (20 mM Tris-HCl/150 mM NaCl, pH 7.4) in the presence of horseradish peroxidase (HRP; 0.03 μM) at 37 °C in the dark. Fluorescence in the range of 490−650 nm (λ_{ex} = 485 nm, λ_{em} = 525 nm) was recorded using a cuvette (1 cm). Cu-cyclen and Cu-cyclam without Aβ40 were used as controls to estimate their interference with the generation of H_2O_2. The signal specific for H_2O_2 was validated by the quenched fluorescence of the parallel sample incubated with catalase (0.02 μM).

Cytotoxic Assay. Cortical neuronal cultures were prepared as described previously with some modifications.[37] Briefly, embryonic day 18.5 C57BL6/J mouse cortices were removed, dissected free of meninges, cut into tissues about 1−2 mm^3, and dissociated in 0.125% (w/v) trypsin in PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter). Cortical neuronal cells were triturated using a filter-plugged fine pipet tip, pelleted, and resuspended in a plating medium (Dulbecco's modified Eagle medium, 10% fetal bovine serum). The cells were plated into poly(L-lysine)-coated 96-well plates at a density of 5000 cells/well in a plating medium. All cultures were maintained in an incubator at 37 °C with 5% CO_2. After 4 h, the plating medium was replaced by a fresh neurobasal medium containing 2% B_{27} supplements, 1% penicillin-streptomycin, and 0.5 mM L-glutamine. Cultures highly enriched with neurons (>95% purity) were thus obtained. The neuronal cells were matured for 6 days in culture before treatment using a freshly prepared serum-free neurobasal medium plus B_{27} supplenments without antioxidants. Solutions of Aβ40 (10 μM), Aβ40 (10 μM) plus Zn^{2+} or Cu^{2+} (20 μM), and Aβ40 (10 μM) plus Zn^{2+} or Cu^{2+} (20 μM) plus cyclen or cyclam (40 μM) were prepared as described above. The cytotoxicity of Aβ40 and Zn- or Cu-Aβ40 with or without chelators toward the cells was assessed quantitatively using the MTT assay.[38] Briefly, neuronal cells were

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treated with each sample solution at 37 °C for 48 h. The medium was replaced with a neurobasal medium supplemented with B_{27} lacking antioxidants (200 μL), and MTT (10 μL, 5 mg/mL) was added to each of the wells, which were incubated at 37 °C for 4 h in an incubator with 5% CO_2. The supernatant was taken off, and DMSO (150 μL) was added to solubilize the MTT formazan. After 20 min of standing and 10 min of shaking, the amount of MTT formazan in each well was determined using a Tecan Sunrise ELISA Reader at 490 nm. The optical density was used to calculate the percentage of cell viability with respect to the untreated control values. The background readings of MTT incubated in a cell-free medium were subtracted from each value before calculation.

Results and Discussion

Effect of Chelators on Aβ Aggregation. The effect of cyclen and cyclam on the Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregation was first assessed by turbidimetry. Turbidity reflects the change in optical density of the solution, and it is an indication of all types of Aβ40 aggregates, including β-aggregates, amorphous aggregates,[39] and so forth. As shown in Figure 1, the turbidity (A_{405}) of the Aβ40 solution alone incubated at 37 °C for 52 h is very small (0.0011 units); however, that of the Aβ40 solution in the presence of Zn^{2+} or Cu^{2+} is considerably increased (0.075 or 0.064 units), indicating that Zn^{2+} and Cu^{2+} promote the aggregation of Aβ40. In contrast with this, the turbidity of the Aβ40 solutions containing both metal ions and additional chelator are only moderately higher than that of Aβ40 alone. The results indicate that Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregation is markedly attenuated by cyclen or cyclam, and the preformed Zn- or Cu-Aβ40 aggregates can be solubilized by these chelators. The Cu^{2+}-induced Aβ40 aggregates appear more susceptible to cyclen or cyclam because Cu^{2+} can form more stable complexes with these chelators than Zn^{2+}.[34,35] A control experiment showed that Zn^{2+} and Cu^{2+} do not form hydroxide precipitates at pH 7.4.

Influence of Chelators on β-Aggregation. The influence of cyclen and cyclam on Zn^{2+}- or Cu^{2+}-induced β-aggregation of Aβ40 was examined by amyloid-specific dye-binding analyses. It is known that the thiazine dye ThT characteristically stains amyloid-like deposits under different conditions.[40] ThT specifically binds to the aggregated β-sheet fibrils common to amyloid structures, and the binding gives rise to a significant enhancement in fluorescence of ThT according to the amount of amyloid.[41] Since the formation of the fluorescent ThT–peptide complex requires the aggregated state of amyloid peptides, the fluorescence variation reflects the structural change of the peptide. As presented in Figure 2, an Aβ40 solution (10 μM) incubated at 37 °C only shows weak fluorescence, indicating that Aβ40 exists primarily in the initial form and the amount of β-aggregates is very low. However, in the presence of Zn^{2+} or Cu^{2+}, the solution displays an enormous increase in fluorescence at 480 nm, suggesting that Aβ40 is transformed from the initial form to the β structure and considerable amounts of β-aggregates are formed. A tremendous decrease in fluorescence

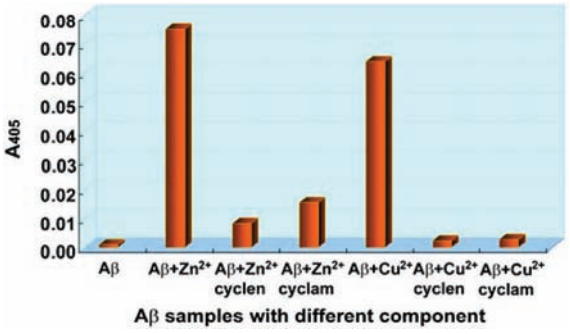

Figure 1. Turbidity (A_{405}) of Aβ40 (10 μM) and Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregates after incubation in the absence and presence of cyclen or cyclam at 37 °C and pH 7.4 for 52 h ([Aβ40]/[metal ion]/[chelator] = 1:2:4).

is observed when cyclen or cyclam is added to the solution of Zn- or Cu-Aβ40, which indicates that these chelators can inhibit Zn^{2+}- or Cu^{2+}-induced β-aggregation of Aβ40. The fluorescence of the Zn-Aβ40 system is relatively stronger than that of the Cu-Aβ40 system because Zn^{2+} is a more potent inducer of such aggregations.[10] The decrease in ThT fluorescence is not due to a structural alteration of the dye or dye–dye interactions in solution during the incubation, since excessive ThT does not induce a marked fluorescence increase under the circumstances.

Changes in Soluble Aβ Peptide. The content of soluble Aβ40 in the supernatant of Aβ40 solution containing Zn^{2+} or Cu^{2+} with or without chelators was determined using the BCA Protein Assay. As Figure 3 shows, most Aβ40 (>86%) is soluble after incubation at 37 °C for 2 days without metal ions and a chelator. However, in the presence of Zn^{2+} or Cu^{2+}, soluble Aβ40 only reaches 13% or 18% of its starting amount, respectively, indicating that most Aβ40 is precipitated by metal ions and Zn^{2+} exerts a greater influence on the solubility of the peptide than Cu^{2+} does. Upon the addition of cyclen or cyclam to the Aβ40 solution preincubated with Zn^{2+} or Cu^{2+}, the content of soluble Aβ40 is largely increased, with the Zn-Aβ40 system reaching 69% or 54% and the Cu-Aβ40 system reaching 85% or 75% of its starting amount, respectively. Cyclen appears to be more effective in promoting the solubilization of Aβ deposits, possibly owing to its smaller size making it easier to approach the metal center of the aggregates. The results demonstrate that the Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregation can be greatly inhibited or reversed by cyclen or cyclam.

The change of soluble Aβ40 in the supernatant of the Aβ40 aggregate solution was further monitored by ESI-MS. In the absence of a chelator, no signal for Aβ40 is found in the ESI-MS spectrum (data not shown). However, in the presence of cyclen or cyclam, the peaks for different Aβ40 species are observed in the spectra (Figure 4). In addition, peaks corresponding to $[Zn(cyclen)Ac]^+$, $[Zn(cyclam)Ac]^+$, $[Cu(cyclen)Ac]^+$, and $[Cu(cyclam)Ac]^+$ are also observed, which confirm the chelation betweem Zn^{2+} or Cu^{2+} with cyclen or cyclam. The results show that cyclen and cyclam are capable of withdrawing Zn^{2+} or Cu^{2+} from the Zn- or Cu-Aβ40 aggregates. The chelators may react directly with the metal center of the aggregates, or they may react with free metal ions in the solution and thereby alter the

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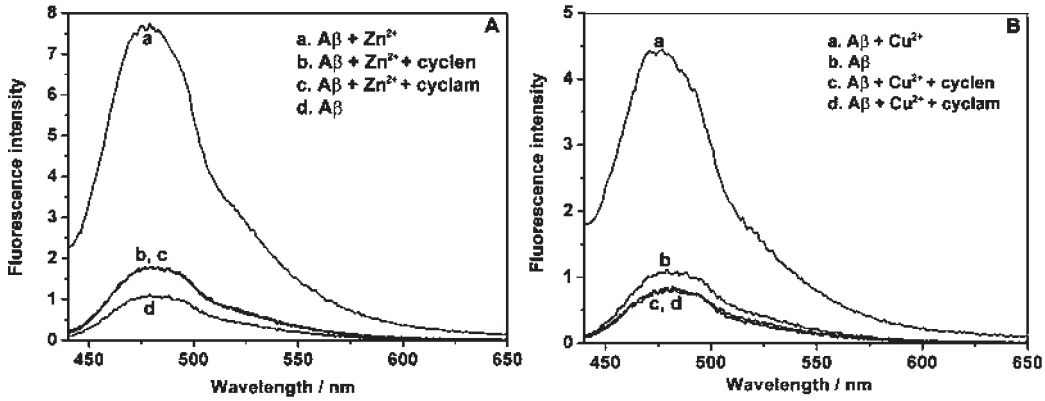

Figure 2. ThT fluorescence spectra (λ_{ex} = 415 nm) of Aβ40 solutions in the absence and presence of Zn^{2+} (A) or Cu^{2+} (B) and cyclen or cyclam after incubation at 37 °C for 52 h ([Aβ40] = 10 μM; [Aβ40]/[metal ion]/[chelator] = 1:2:4).

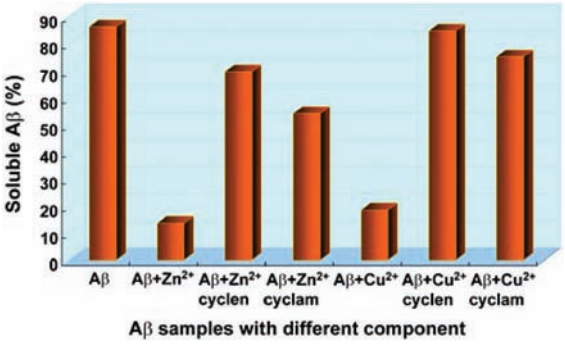

Figure 3. The percentage of soluble Aβ40 in the solution containing Zn^{2+} or Cu^{2+} with or without chelators after incubation at 37 °C for 52 h ($[A\beta40]_{initial}$ = 100 μM; [metal ion] = 200 μM; [chelator] = 400 μM).

equilibrium between metal ions and precipitates. Both actions lead to the dissolution of Aβ40 aggregates. Assignments of the observed peaks in the ESI-MS spectra are summarized in Table 1.

It was reported recently that some cyclen derivatives and their metal complexes can cleave soluble oligomers of Aβ into fragments;[42,43] however, cyclen, cyclam, and the subsequently formed metal complexes do not show obvious cleavage activity toward the Aβ aggregates. In the above ESI-MS spectra, monomers with different charges are the dominant species after the solubilization. Some minor unidentifiable signals might result from the fragments of Aβ monomers, but further evidence is needed to verify this presumption.

Variations in Metal Content. The content of metal ions remaining in the supernatant of Aβ40 aggregate solution after treatment with or without chelators was measured by ICP-MS. As shown in Table 2, in the supernatant of Zn- or Cu-Aβ40 solution without a chelator, the content of Zn^{2+} or Cu^{2+} is very low; in the presence of cyclen or cyclam, however, the metal content is significantly increased. The results indicate that Zn^{2+} or Cu^{2+} ions have been extracted from Zn- or Cu-Aβ40 aggregates by the chelators to become soluble metal complexes. Cyclen seems more effective than cyclam in grabbing Zn^{2+} from Zn-Aβ40 aggregates, but in the case of Cu-Aβ40 aggregates, an opposite order is observed. This result can be explained by the related stability constant of the complexes, since $K_{Zn\text{-cyclen}}$ is larger than $K_{Zn\text{-cyclam}}$ while $K_{Cu\text{-cyclen}}$ is less than $K_{Cu\text{-cyclam}}$.[33−35]

Restoration of Aβ Conformation. It has been shown that AD arises from protein conformational disorder (PCD), that is, protein misfolding, and a change in the secondary or tertiary structure of a normal protein without alteration of the primary structure is the hallmark in PCD.[44] In most PCDs, the misfolded protein is rich in β-sheet conformation.[45] In AD, for example, a conformational change from a random coil to a β-sheet structure is necessary for Aβ fibril formation, and this change is promoted by the presence of metal ions that accelerate the aggregation of Aβ from solution.[46,47] The impact of cyclen or cyclam on the change of secondary structure of Aβ40 in the presence of Zn^{2+} or Cu^{2+} was thus investigated by CD spectroscopy. As Figure 5 shows, Aβ40 freshly dissolved in a buffer exhibits a typical CD spectrum of the random coil conformation.[46] However, in the presence of Zn^{2+} or Cu^{2+}, a characteristic spectrum for a β-sheet structure appears with a minimum ellipticity at 216 nm. The results are consistent with the above observed metal effects on β-aggregation monitored by ThT fluorescence; nevertheless, they are inconsistent with the previous hypothesis that the conformational state of Aβ is not perturbed by precipitation with Zn^{2+}.[18] After the addition of cyclen or cyclam to the Zn- or Cu-Aβ40 system, the spectrum typical of the random coil conformation reappears and overlaps that of Aβ40. These observations suggest that Zn^{2+} or Cu^{2+} has been removed by the chelators from the aggregates, and the peptide is reverted to a random coil conformation from the β-sheet structure. Therefore, cyclen and cyclam not only solubilize Zn^{2+}- or Cu^{2+}-induced Aβ40 aggregates but also restore the conformation of the peptide.

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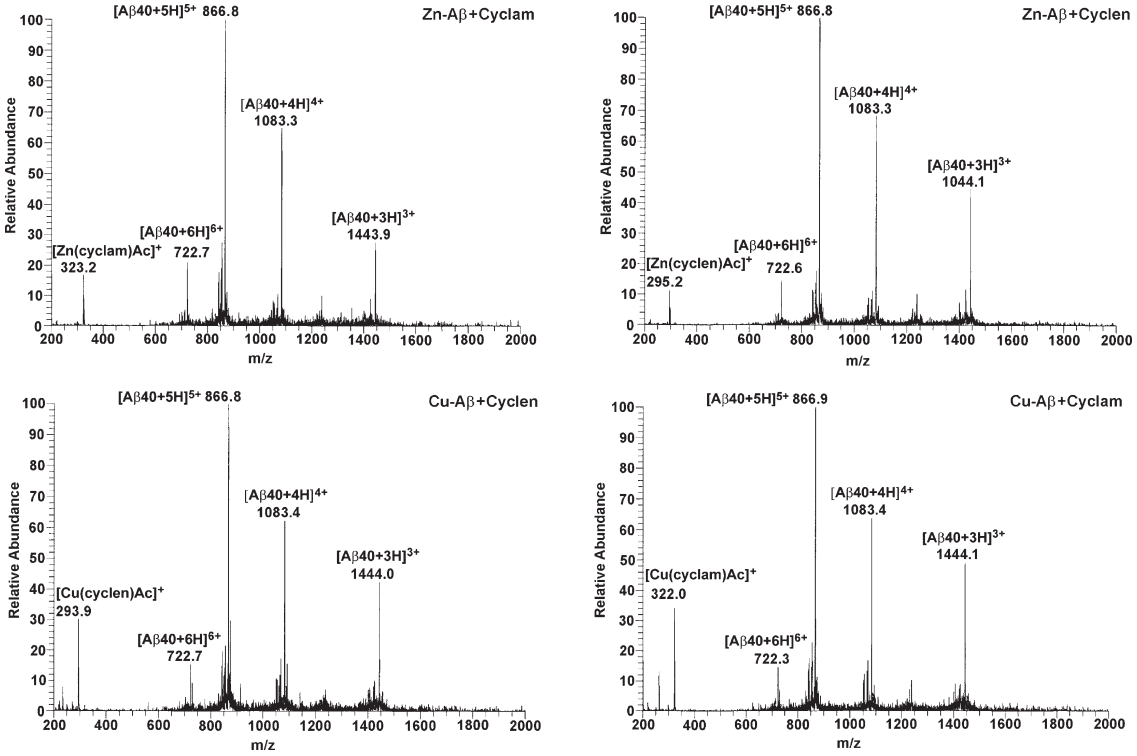

Figure 4. ESI-MS spectra (positive ion mode) of the supernatant of Zn- or Cu-Aβ40 aggregate solutions with or without cyclen or cyclam ($[A\beta40]_{initial}$ = 100 μM; [metal ion] = [chelator] = 200 μM).

Table 1. Assignments of the Major Observed Peaks in the ESI-MS Spectra for Different Aβ40 Species and Metal−Chelator Complexes (see Figure 4)

species	formula	obsd m/z	calcd m/z
$[A\beta40 + 6H]^{6+}$	$C_{194}H_{301}N_{53}O_{58}S$	722.3−722.7	722.7
$[A\beta40 + 5H]^{5+}$	$C_{194}H_{300}N_{53}O_{58}S$	866.8−866.9	867.0
$[A\beta40 + 4H]^{4+}$	$C_{194}H_{299}N_{53}O_{58}S$	1083.3−1083.4	1083.5
$[A\beta40 + 3H]^{3+}$	$C_{194}H_{298}N_{53}O_{58}S$	1443.9−1444.1	1444.3
$[Zn(cyclen)Ac]^+$	$C_{10}H_{23}N_4O_2Zn$	295.2−300.0	296.7
$[Zn(cyclam)Ac]^+$	$C_{12}H_{27}N_4O_2Zn$	322.0−323.2	324.8
$[Cu(cyclen)Ac]^+$	$C_{10}H_{23}N_4O_2Cu$	293.9−296.0	294.9
$[Cu(cyclam)Ac]^+$	$C_{12}H_{27}N_4O_2Cu$	322.0−325.1	322.9

Table 2. Content of Metal Ions in the Supernatant of Zn- or Cu-Aβ40 Solution with or without Cyclen or Cyclam Detected by ICP-MS ($[A\beta40]_{initial}$ = 100 μM; [Aβ40]/[metal ion]/[chelator] = 1:2:4)

sample	soluble Zn (mg/L)	sample	soluble Cu (mg/L)
Aβ + Zn^{2+}	0.019	Aβ + Cu^{2+}	0.048
Aβ + Zn^{2+} + cyclen	0.306	Aβ + Cu^{2+} + cyclen	0.319
Aβ + Zn^{2+} + cyclam	0.291	Aβ + Cu^{2+} + cyclam	0.323

The native and active conformation of Aβ is a mixture of α-helical and random structure, while the aggregated Aβ adopts a β-sheet conformation. β-sheets are formed from alternating peptide pleated strands linked by hydrogen bonds between the NH and CO groups of the peptide bond. In α-helices, the hydrogen bonds are between groups within the same strand, but in β-sheets, the bonds are between one strand and another. Since the second strand can come from a different region of the same protein or from a different molecule, the β-sheet structure is usually stabilized by oligomerization or aggregation.[48]

Cyclen and cyclam extract Zn^{2+} or Cu^{2+} from the Aβ aggregates and thereby destabilize the β-sheet structure and induce its conversion into the normal form. Considering that the Aβ aggregation is central in the pathogenesis of AD, chelators that can inhibit or reverse the conformational changes of Aβ should have high therapeutic potential.

Protective Capability of the Chelators. The protective effect of cyclen and cyclam on soluble Aβ40 was studied by BCA assay. As Figure 6 and Table 3 present, in the absence of chelators, Zn^{2+}- or Cu^{2+}-induced aggregation is very prominent, and only less than half of Aβ40 is soluble. However, in the presence of chelators, Zn^{2+}- or Cu^{2+}-induced aggregation is largely inhibited. When the molar ratio of the chelator to Aβ40 reaches 2:1, the metal-induced aggregation is almost completely prevented. At the same molar ratio, cyclen demonstrates a more powerful potentiality than cyclam to prevent the peptide from aggregation. Thus, cyclen and cyclam may serve as protectants to prevent the formation of Aβ40 aggregates.

Selectivity of the Chelators. Ideal chelators should have an intermediate affinity and be capable of disrupting low-affinity but pathologically relevant Aβ−metal interactions without disrupting high-affinity essential protein/peptide−metal interactions.[49] In other words, the chelation of excess metals in the brain of AD patients should be specific rather than systemic. However, no available chelator is exclusively specific for any particular metal ion. Therefore, whether a chelator is advisable to be a possible therapeutic agent for AD largely depends on its affinity for different metal ions. The selective affinities of cyclen and cyclam for Zn^{2+} and

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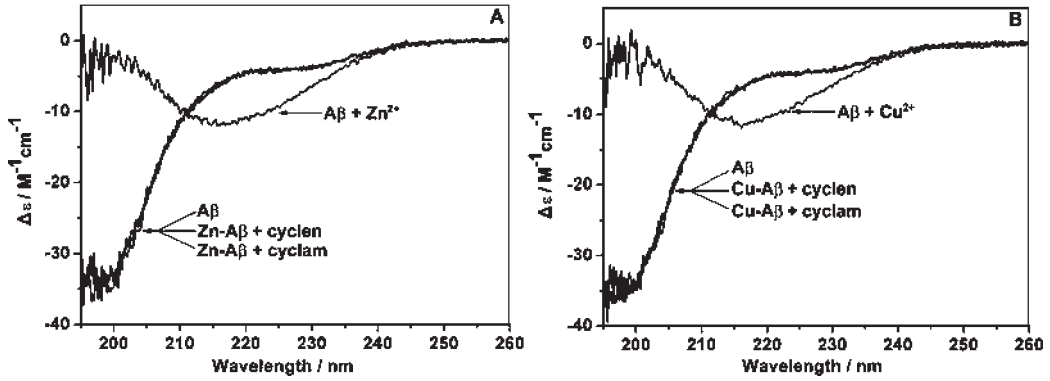

Figure 5. Circular dichroism spectra of Aβ40 alone or in the presence of Zn^{2+} (A) or Cu^{2+} (B) before and after addition of cyclen or cyclam at 37 °C ($[A\beta40]_{initial}$ = 50 μM; [Aβ40]/[metal ion]/[chelator] = 1:2:4).

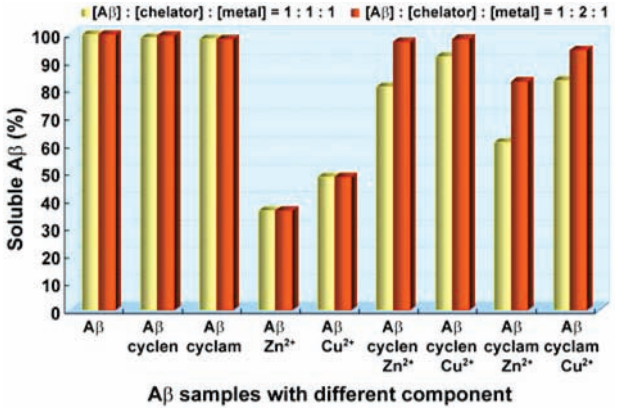

Figure 6. Percentage of soluble Aβ40 in the absence and presence of cyclen or cyclam before and after addition of Zn^{2+} or Cu^{2+} ([Aβ] = [metal] = 100 μM).

Table 3. Concentration of Soluble Aβ40 in the Absence and Presence of Cyclen or Cyclam before and after Addition of Zn^{2+} or Cu^{2+} and Incubation at 37 °C for 4 h

sample	soluble Aβ (μg/mL) (Aβ %)	
[Aβ] = [metal] = 100 μM	[Aβ]/[chelator] = 1:1	[Aβ]/[chelator] = 1:2
Aβ	433.0 (100)	433.0 (100)
Aβ + cyclen	427.7 (98.8)	431.1 (99.6)
Aβ + cyclam	426.2 (98.4)	425.2 (98.2)
Aβ + Zn^{2+}	156.3 (36.1)	156.3 (36.1)
Aβ + Cu^{2+}	208.9 (48.2)	208.9 (48.2)
Aβ + cyclen + Zn^{2+}	350.4 (80.9)	421.5 (97.3)
Aβ + cyclen + Cu^{2+}	397.9 (91.9)	425.8 (98.3)
Aβ + cyclam + Zn^{2+}	263.4 (60.8)	359.1 (82.9)
Aβ + cyclam + Cu^{2+}	360.6 (83.3)	408.7 (94.4)

Cu^{2+} relative to more abundant Ca^{2+} and Mg^{2+} were thus surveyed in a simulated situation, since Ca^{2+} and Mg^{2+} were found to be involved in the chelator-mediated release of Aβ from Zn- or Cu-Aβ aggregates.[50] As shown from the data obtained using a BCA protein assay (Table 4), in the absence of cyclen or cyclam, the aqueous solubility of Aβ40 is dramatically decreased by the presence of Zn^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+} as compared with that without metal ions; in the presence of chelators, however, a large portion of Aβ40 becomes soluble. The results indicate that the selective

Table 4. Solubility of Zn^{2+}- and Cu^{2+}-Induced Aβ Aggregates in the Presence of Ca^{2+} and Mg^{2+} with or without Cyclen or Cyclam at 37 °C in Water Determined by BCA Protein Assay Kit ([Aβ40] = [Zn^{2+}] = [Cu^{2+}] = 100 μM, [Ca^{2+}] = [Mg^{2+}] = 2 mM, [chelator] = 400 μM)

sample	Aβ (μg/mL)	soluble Aβ (%)
Aβ	433.0	100
Aβ + Zn^{2+} + Cu^{2+}	55.7	13
Aβ + Zn^{2+} + Cu^{2+} + Ca^{2+} + Mg^{2+}	34.7	8
Aβ + Zn^{2+} + Cu^{2+} + Ca^{2+} + Mg^{2+} + cyclen	379.8	88
Aβ + Zn^{2+} + Cu^{2+} + Ca^{2+} + Mg^{2+} + cyclam	281.4	65

affinities of cyclen and cyclam for Zn^{2+} and Cu^{2+} are barely influenced by other essential metal ions, and the chelators are effective in Zn^{2+}- or Cu^{2+}-induced Aβ aggregates, even in the presence of Ca^{2+} and Mg^{2+}. This effect is expectable because the affinity of these chelators for Ca^{2+} and Mg^{2+} is quite weak; for example, the stability constant (log *K*) of Ca−cyclen and Mg−cyclen only reaches 3.12 and 2.25, respectively.[51] Therefore, even if the chelator becomes occupied by Ca^{2+} or Mg^{2+} en route to the Aβ aggregates, the ultimate ionic exchange for Cu^{2+} or Zn^{2+} into the chelating ring at the metal binding sites in peptide is still doomed to happen. Accordingly, these chelators are supposed to merely coordinate Zn^{2+} and Cu^{2+} in the cerebral amyloid mass without systemically sequestering Ca^{2+} and Mg^{2+}.

Effect of Chelators on the Morphology of Aβ Aggregates. Morphological analysis of Aβ aggregates on the surface of mica formed by interaction between Aβ40 and Zn^{2+} or Cu^{2+} with or without a chelator was achieved by ex situ AFM. As Figure 7 shows, in the presence of Zn^{2+} (A) or Cu^{2+} (B) without a chelator, only nonuniform and nonfibrillar amorphous aggregates are observed. It was found that Zn^{2+} and Cu^{2+} can dissociate most fibrillar Aβ aggregates at high concentrations (~100 μM) to yield the amorphous form.[52] Therefore, no fibrillar aggregates appear in the AFM image, which is consistent with the case reported for Aβ42.[39] The observation suggests that Zn- or Cu-Aβ40 aggregates may not be central mediators that induce fibrillar amyloid plaques in AD. Interestingly,

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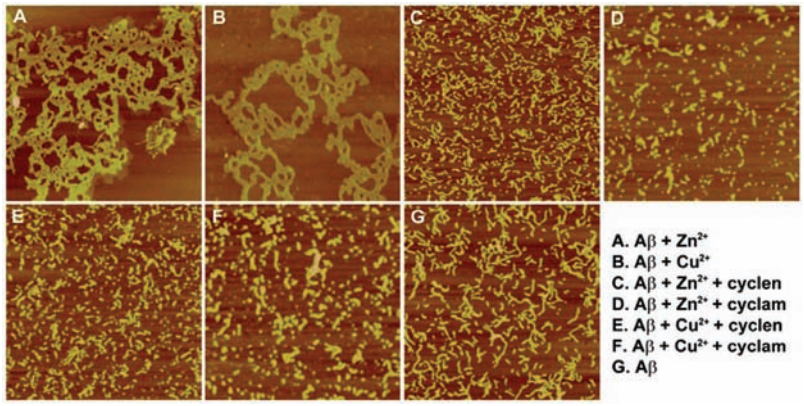

Figure 7. Representative AFM images (2 μm × 2 μm) of metal-Aβ40 deposits formed on the surface of mica under ambient conditions. Aβ40 deposits containing Zn^{2+} (A, C, D) or Cu^{2+} (B, E, F) with and without cyclen or cyclam were prepared by immobilizing Aβ40 sample solutions on mica ([Aβ40] = 50 μM, [metal ion] = 100 μM, [chelator] = 200 μM). Aβ40 (G) is taken as a control.

Table 5. Mean Height and Width of the Deposits on the Surface of Mica Obtained from AFM Images (Figure 7)[a]

sample	image	height (nm)	width (nm)
Aβ + Zn^{2+}	A	2.18	65.78
Aβ + Cu^{2+}	B	1.44	71.78
Aβ + Zn^{2+} + cyclen	C	3.17	33.78
Aβ + Zn^{2+} + cyclam	D	3.06	46.78
Aβ + Cu^{2+} + cyclen	E	3.14	38.67
Aβ + Cu^{2+} + cyclam	F	3.24	52.67
Aβ	G	3.00	39.89

[a] Each image was divided into nine regions, and five spots were picked randomly for datum analysis.

the morphologies of Aβ40 aggregates induced by Zn^{2+} and Cu^{2+} respectively are somewhat different. As the quantitative distribution analysis indicates (Table 5), the mean height of the Aβ40 aggregates induced by Zn^{2+} is much larger than that induced by Cu^{2+} (2.18 vs 1.44 nm), and the mean width of the former is smaller than that of the latter (65.78 vs 71.78 nm). In addition, the amounts of Zn-Aβ40 aggregates formed on the mica surface are greater than that of Cu-Aβ40 aggregates. The results indicate that Zn^{2+} induces the formation of Aβ40 aggregates at a much higher strength than Cu^{2+}, which agrees with the results discussed above.

In the presence of cyclen or cyclam, the morphology of the Aβ40 deposits on the mica surface changed a lot as compared to that without chelators. Large plaques of amorphous Aβ40 aggregates disappeared, and rodlike or globular deposits are observed throughout the surface (Figure 7C−F). The length of the rods varies from 100 to 600 nm, with the average length being ca. 100−200 nm. The images of Aβ40 aggregates coexisting with Zn^{2+} (C, D) or Cu^{2+} (E, F) and cyclen or cyclam are quite similar to that of Aβ40 alone (G), especially those in the presence of cyclen (C, E). The quantitative analysis of these images in terms of height shows that the mean thickness of Aβ40 deposits on the surface of mica is about 3 nm (Table 5). These results demonstrate that both cyclen and cyclam disrupt the formation of amorphous Aβ40 aggregates and reverse the preformed aggregates to the original form of Aβ40. Aβ40 per se can form some kind of aggregate spontaneously because of the increased attractive force between the molecules under proper conditions.[53]

Inhibition of H_2O_2 Generation. H_2O_2 is the substrate for the Fenton reaction that generates the highly reactive hydroxyl radical ($OH^{\bullet}$). Much evidence has indicated that oxidative injury in AD is mediated by H_2O_2.[54] H_2O_2 permeates freely across all tissue boundaries and reacts with reduced metal ions (Fe^{2+}, Cu^{+}) to generate $OH^{\bullet}$. This species in turn engenders lipid peroxidation adducts, protein carbonyl modifications, and nucleic acid adducts in all cellular compartments, which typifies the neuropathology of AD.[55,56] When Cu^{2+} binds to Aβ, a series of electron transfer reactions occurs, including the reduction of Cu^{2+} to Cu^{+}, and thereby results in the formation of H_2O_2.[57,58] This process is catalytic in that biological reducing agents such as ascorbate and dopamine are involved, which has been implicated in the neurotoxicity of Aβ in AD.[59]

The influence of cyclen and cyclam on the production of H_2O_2 mediated by Aβ40 in the presence Cu^{2+} was determined using a DCF assay. H_2O_2 oxidizes nonfluorescent DCFH to highly fluorescent DCF, and the oxidation demonstrates good linearity.[60] Therefore, the production of H_2O_2 can be evaluated from the fluorescence intensity of DCF. HRP is used in the assay to catalyze the reaction of H_2O_2 with DCFH since Cu-Aβ alone does not increase DCF fluorescence.[59] As Figure 8 shows, the fluorescence intensity of DCF is quite strong for the Cu-Aβ40 system without a chelator (a), suggesting that the generation of

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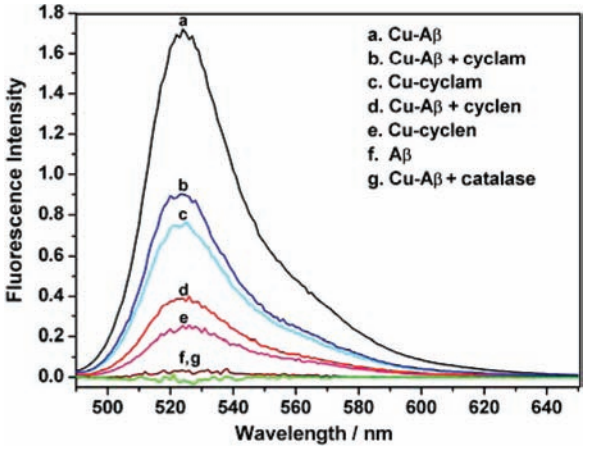

Figure 8. Fluorescence of DCF (λ_{ex} = 485 nm, λ_{em} = 525 nm) reflecting the effect of cyclen and cyclam on the production of H_2O_2 from Aβ40 in the presence of Cu^{2+} ([Aβ40] = 300 nM, [Cu^{2+}] = [Cu-cyclen] = [Cu-cyclam] = 150 nM, [HRP] = 0.03 μM, [DCFH] = 100 μM, [ascorbate] = 2 μM, [catalase] = 0.02 μM, pH = 7.4).

H_2O_2 is unrestrained. In the presence of cyclen or cyclam, the corresponding fluorescence intensity decreases apparently; however, the system still shows much fluorescence (b, d). Control experiments demonstrate that Cu−cyclam and Cu−cyclen complexes without Aβ40 also generate considerable H_2O_2 under this condition (c, e). Therefore, the remnant fluorescence may mainly arise from the in situ formed copper complex after the chelation. Under the circumstances, the actual inhibition effect of cyclen and cyclam on Aβ-mediated H_2O_2 formation should be greater than that indicated by the apparent curves. In other words, the H_2O_2 induced by the Cu-Aβ40 aggregate can be largely abolished by these chelators. Cyclen appears more potent than cyclam in suppressing the oxidation process, but if the interference of the Cu−amine complex is taken into consideration, the superiority is rather slim. Finally, some Cu^{2+} ions bound to the high-affinity sites of Aβ40 may also contribute their share to the residual fluorescence. As expected, Aβ40 alone does not produce H_2O_2.

The Cu-Aβ40-induced oxidation of DCFH is almost completely inhibited by catalase because of the decomposition of H_2O_2 by this enzyme (Figure 8, line e). The result verifies that the fluorescence of DCF observed in the above assays is the consequence of the production of H_2O_2 in the system rather than some other cause. Cyclen and cyclam may work by a combined action that facilitates the disaggregation of Cu-Aβ40 aggregates on one side and inhibits the production of H_2O_2 on the other side. Therefore, the interdiction of Cu^{2+} interactions with Aβ by these chelators may promote the clearance of Aβ aggregates and mitigate the neurotoxic effect exerted by H_2O_2 in AD treatment.

Influence of Chelators on the Cytotoxicity of Aβ Aggregates. The cytotoxicity of Zn- or Cu-Aβ40 aggregates in the absence and presence of cyclen or cyclam toward cortical neuronal cells of C57BL6/J mice was examined using the MTT assay, and the results are shown in Figure 9. Aβ40 alone only shows a marginal toxicity toward the neuronal cells, and the cell viability remains larger than 90% as compared with the control value (100%). The toxicity of Aβ40 preincubated with Zn^{2+} or Cu^{2+} is much higher than that of Aβ40, with the cell

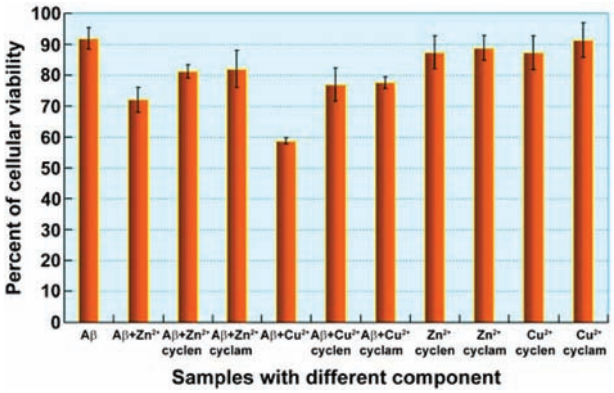

Figure 9. Cytotoxicity of Aβ40 and Zn- or Cu-Aβ40 aggregates with and without cyclen or cyclam toward the cortical neuronal cells of C57BL6/J mice after incubation for 48 h ([Aβ40] = 10 μM; [Aβ40]/[metal]/[chelator] = 1:2:4).

viability declining apparently, especially for the culture containing the Cu-Aβ40 aggregate (< 60%). The toxicity of the metal-Aβ40 aggregates is obviously attenuated in the presence of cyclen or cyclam, with the cell viability rising to around 80%. The neuronal cells seem more sensitive to the alteration of the Cu-Aβ40 aggregate than to that of the Zn-Aβ40 aggregate, since the former is correlated with the generation of H_2O_2 as noted above. The cytotoxicity of the metal−chelator complexes was also tested under the same condition. The viability of the neuronal cells is comparable to that observed for Aβ40 alone, suggesting that the toxicity under discussion mainly arises from the metal-Aβ40 aggregates. These results show that both cyclen and cyclam can suppress the cytotoxicity of Zn- or Cu-Aβ40 aggregates to some extent and consequently enhance the viability of neuronal cells.

Conclusion

Metal chelators are promising therapeutic agents for Alzheimer's disease. In this study, we have investigated the effect of two chelators, cyclen and cyclam, on the aggregation of Aβ induced by Zn^{2+} and Cu^{2+}. These chelators exhibit prominent abilities to dissolve the metal-induced Aβ aggregates and to protect Aβ from precipitation in the presence of Zn^{2+} and Cu^{2+}, and they also prevent the formation of the β-sheet structure and promote the reversion of the β-sheet to the normal random coil conformation. Moreover, cyclen and cyclam inhibit the production of H_2O_2 induced by Cu-Aβ40 and thereby palliate the toxicity of the aggregates. The merits of cyclen and cyclam are further glorified by some of other inherent properties, such as a low molecular weight, poor or noncharged form, and amphiphilic solubility. All of these properties are beneficial for them to cross the blood−brain barrier. Considering the above advantages and low toxicity of cyclen and cyclam, these chelators deserve further investigation or optimization as potential therapeutic agents for Alzheimer's disease.

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