Decelerated degradation of β-amyloid (Aβ) and its interaction with synaptic copper may be pathogenic in Alzheimer disease. Recently, Co(III)-cyclen tagged to an aromatic recognition motif was shown to degrade Aβ in vitro. Here, we report that apocyclen attached to selective Aβ recognition motifs (KLVFF or curcumin) can capture copper bound to Aβ and use the Cu(II) in place of Co(III) to become proteolytically active. The resultant complexes interfere with Aβ aggregation, degrade Aβ into fragments, preventing H₂O₂ formation and toxicity in neuronal cell culture. Because Aβ binds Cu in amyloid plaques, apocyclen-tagged targeting molecules may be a promising approach to the selective degradation of Aβ in Alzheimer disease. The principle of copper capture could generalize to other amyloid-oses where copper is implicated.

β-Amyloid (Aβ) is a toxic 39–43-residue polypeptide that accumulates in the brain in Alzheimer disease (AD) as a result of an imbalance between Aβ production and degradation (1–3). Although the precise mechanisms by which Aβ exerts its toxic effects on neurons remain to be fully elucidated, there is evidence that Aβ neurotoxicity involves forming pathological assemblies of the peptide and evoking the generation of free radicals by binding and reducing redox-active metals such as copper (Cu(II)) (3–5).

The decelerated degradation of Aβ is likely to be a major factor in AD (2, 3). This degradation has been proposed to involve several enzymes, including nephrilysin (6, 7), insulin-degrading enzyme (3), and cathepsin B (8). The activity and expression of these enzymes appear to decline in the early stages of AD progression and with aging (9). However, it is difficult to control the activation of these enzymes pharmacologically (9).

Aβ can bind to and reduce Cu(II) to generate toxic reactive oxygen species (10, 11), Aβ oxidation, and cross-linking (12). Copper is concentrated in amyloid plaques (≈0.4 μM) (13) and directly coordinates Aβ at the histidine side chains in its amino terminus (11, 14).

It has been suggested that Aβ aggregation blockers (15) and copper chelators (16) could have therapeutic benefits for AD. Recently, a novel approach to Aβ clearance has been reported which involves the degradation of Aβ by a small lytic molecule, Co(III)-cyclen linked to an aromatic oligomer-recognition molecule (17). The Co(III)-cyclen complex hydrolyzes peptide bonds. We recognized that apocyclen (cyc) would be inert yet become activated, and prevent both Aβ oligomerization and toxicity as well as cleave the peptide. This multifunctional approach yields a potent and selective strategy for the clearance of Aβ.

**EXPERIMENTAL PROCEDURES**

*Preparation of Cyc(Cu(II))-KLFF and Other Complexes—Cyclen-hybrid cleavage agents were synthesized by routine synthetic methods, as detailed in supplemental “Experimental Procedures” and supplemental Fig. S1. The stock solutions of cyc(Cu(II))-KLFF and other Cu(II) complexes were prepared by adding an aqueous solution of CuCl₂ (1 eq) to the com-

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental “Experimental Procedures,” Table S1, and Fig. S1.

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* The abbreviations used are: Aβ, β-amyloid; AD, Alzheimer disease; Cur, curcumin; cyc, apocyclin; fAβ, fibrillar β-amyloid; HFPi, 1, 1, 1, 3, 3, 3-hexafluoroisopropanol; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyterazoliumbromide; nAβ, oligomeric β-amyloid; PBS, phosphate-buffered saline; TCEP, tri(2-carboxyethyl)phosphine hydrochloride; TEM, transmission electron microscopy; ThT, thioflavin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.
Cu-Cyclen Cleavage of Aβ

**A**

\[ \text{Cyc(KLVFF)} \rightarrow \text{Cu(II)} \rightarrow \text{Cyc(KLVFF)} \]

**B**

**C**

**D**

**FIGURE 1.** Cyc-KLVFF sequesters Cu(II) from Aβ and inhibits the generation of H$_2$O$_2$. A, chemical structures of cyc-KLVFF and cyc(Cu(II))-KLVFF. B, competitive effect of cyc-KLVFF on Aβ for Cu(II), as measured by tyrosine intrinsic fluorescence. Fresh Aβ(1–42) (10 μM) was incubated alone or with Cu(II) (16 μM) or cyc-KLVFF (20 μM) together for 3 h. Data points are means ± S.D., n = 3. C, cyc-KLVFF rescues Cu(II) from Aβ–Cu(II) complex. After fresh Aβ(1–42) (10 μM) was incubated with Cu(II) (16 μM) for 2 h, cyc-KLVFF (40 μM) was added to the solution, and the mixture was incubated for a further hour. All data points are means ± S.D., n = 3. D, inhibition of H$_2$O$_2$ generated by Aβ1–42–Cu(II) (10 μM) complexes by cyc-KLVFF (concentrations indicated). Data are means ± S.D., n = 3. **p < 0.01.

Compounds (1.2 eq). The stock solutions were incubated at 37 °C for 4 h before use.

**Fresh Aβ Stock Solution Preparation—Aβ(1–42) (American Peptide) or Aβ(1–40) (gift from R. Cherny) were solubilized to 2 mg/ml in 1,1,1,3,3,3-hexafluoropropanol (HFIP; Acros), incubated overnight at room temperature, and stored at −80 °C in HFIP. Aliquots of this solution were evaporated off under vacuum and then dissolved in 20 mM NaOH sonicated for 15 min. The solution was diluted 1:10 with Millipore water and 1:10 with Millipore water and phosphate-buffered saline (PBS) (pH 7.4), centrifuged for 10 min at 20,000 × g, and the supernatant (fresh Aβ stock solution) was further diluted with PBS (pH 7.4) to initiate fibrillogenesis. Before use, all buffers and solutions were passed through a 0.20-μm filter to remove any particulate matter. The concentrations of stock Aβ peptide were determined by spectrophotometric absorbance at 214 nm (against calibrated standard curves). Aβ fresh stock solution was utilized for hydrogen peroxide assay, thioflavin T (ThT) fluorescence, transmission electron microscopy (TEM), Western blot analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazoliumbromide (MTT) assay and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay.

**Fibrillar Aβ (fAβ) Preparation—Fresh Aβ(1–42) (10 μM) was incubated in PBS (pH 7.4) at 37 °C for 2 days and monitored by ThT assay and TEM.**

**Oligomeric Aβ (oAβ) Preparation—Soluble Aβ(1–42) oligomers were prepared by dissolving 1.0 mg of Aβ in 400 μl of HFIP overnight at room temperature (21). 100 μl of the resulting seedless Aβ solution was added to 900 μl of Millipore water in an Eppendorf tube. After a 10–20-min incubation at room temperature, the samples were centrifuged for 15 min at 14,000 × g, and the supernatant fraction (pH 2.8–3.5) was transferred to a new tube and subjected to a gentle stream of N₂ for 5–10 min to evaporate the HFIP. The samples were then stirred at 500 rpm for 24–48 h at room temperature.

**Tyrosine Intrinsic Fluorescence—Fluorescence spectra were collected using a Hitachi FP-4500 fluorescence spectrophotometer (22, 23). An excitation frequency of 280 nm (slit width, 5 nm) was used, and data were collected over 290–400 nm (slit width, 5 nm). Samples were placed in a four-sided quartz fluorescence cuvette, and data were recorded at room temperature.**

**Hydrogen Peroxide Assay—The colorimetric H$_2$O$_2$ assay was performed in a 96-well microtiter plate (Amresco), according to an existing protocol (10). Fresh Aβ(1–42) (10 μM), Cu-Gly (2 μM; 16 μM), cyc-KLVFF, or other compounds (concentrations as indicated in the figures), and a H$_2$O$_2$ scavenging agent, tri(2-carboxyethyl)phosphine hydrochloride (TCEP, 50 μM; Alfa Aesar) were coincubated in PBS (pH 7.4) for 1 h at 37 °C. After incubation, the unreacted TCEP was detected by 5,5'-dithiobis(2-nitrobenzoic acid) (50 μM; Sigma). The amount of H$_2$O$_2$ was proportional to the absolute absorbance difference between a sample and catalase-only (1,000 units/ml; Sigma) control at 405 nm.**

**ThT Assay—ThT assay was performed by combining 20 μl of incubated solution with a 700-μl solution of 10 μM ThT in 12 mM phosphate buffer (pH 7.4). Fluorescence measurements were performed in a 96-well microtiter plate (Amresco), according to an existing protocol (10). Fresh Aβ(1–42) (10 μM), Cu-Gly (2 μM; 16 μM), cyc-KLVFF, or other compounds (concentrations as indicated in the figures), and a H$_2$O$_2$ scavenging agent, tri(2-carboxyethyl)phosphine hydrochloride (TCEP, 50 μM; Alfa Aesar) were coincubated in PBS (pH 7.4) for 1 h at 37 °C. After incubation, the unreacted TCEP was detected by 5,5'-dithiobis(2-nitrobenzoic acid) (50 μM; Sigma). The amount of H$_2$O$_2$ was proportional to the absolute absorbance difference between a sample and catalase-only (1,000 units/ml; Sigma) control at 405 nm.
were recorded in a Hitachi FP-4500 fluorescence spectrometer at room temperature using a 1-cm path length quartz cell. The excitation wavelength was set to 440 nm (slit width, 5 nm), and emission was monitored from 450 to 600 nm (slit width, 5 nm). The percent aggregation is arbitrarily defined at baseline (0%) by the fluorescence of a solution of ThT to which no Aβ(1–42) was added and at maximum (100%) by the fluorescence after a 2-day incubation of Aβ(1–42) alone. A linear relationship between ThT fluorescence and percent aggregation is used for simplicity.

**TEM**—The TEM samples were prepared by placing 8 μl of the incubated solution monitored by ThT assay on 300-mesh formvar-coated copper grids for 2 min before removing excess solution. Then the sample was stained with 1% fresh tungstophosphoric acid for another 2 min. The grid was blotted on filter paper and allowed to dry before observing the specimen in a JEOL-1200EX electron microscope (JEOL) at 100 kV.

**Western Blot Analysis**—The samples were boiled with sample buffer for 5 min and electrophoresed on a 10–20% SDS/polyacrylamide gel using a Tris/Tricine buffer system. Then the gel was transferred onto membrane, blocked with milk (10% w/v) and probed with WO2 antibody. The membrane was incubated with a secondary polyclonal rabbit anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences) and developed by the ECL detection system (Amersham Biosciences).

**MALDI-TOF/MS**—Samples were first passed through a reverse phase C18 Zipitp (Eppendorf) to remove salts, according to the manufacturer’s instructions, then diluted 1:1 with matrix solution (a saturated solution of 3, 5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% trifluoroacetic acid in water), loaded onto a plate, and allowed to dry. The sample was then analyzed on an Applied Biosystems 4700 Proteomics analyzer MALDI-TOF/TOF operated in reflection mode. Myoglobin was used as an internal standard.

**Neurotoxicity Assay**—Two kinds of cells were studied for the ability of the hybrid compounds to be tolerated in culture and to rescue Aβ toxicity. The mouse neuroblastoma N2a cells were a gift from H. Xu. The cells were plated in 24-well plates (Corning Costar Corporation) and then incubated for 28 h at 37 °C. We added 10 μM Cu(II) after 4 h from plating, and the medium was changed to fresh minimal Eagle’s medium without serum before commencing treatment. Fresh Aβ(1–42) (100 μM) was incubated for 8 h in minimal Eagle’s medium at 37 °C before the experiment. Cyc-KLVFF (from 1 to 20 μM) and Aβ (5 μM) were added into the wells, respectively. MTT was then added to each well, and the absorbance of the colored formazan product was measured at 490 nm. The rat hippocampal neurons were prepared as described (24). Fresh Aβ(1–42) (40 μM) was incubated with or without cyclen-hybrid cleavage agents (40 μM) for 3 days at 37 °C in PBS (pH 7.4) and then diluted 1:4 with culture medium and applied to neurons. The neurons were incubated for 2 days at 37 °C and 5% CO2, and thereafter the cell viability was evaluated using a MTT assay.

**Immunofluorescence Microscopy**—Rat hippocampal neurons were treated the same as for the neurotoxicity assays. Fresh Aβ(1–42) (100 μM) was incubated for 8 h in minimal Eagle’s medium at 37 °C before the experiment. The final concentration of Aβ was 1 μM, and cyc(Cu(II))-KLVFF was present at 10 μM. After a 2-day incubation, cells were prepared for microscopy as described (24).

**RESULTS**

Cyc-KLVFF Captures Cu(II) to Disrupt Aβ-Cu(II) Aggregation and Inhibit Generation of H2O2. Cu(II) binding to Aβ quenches tyrosine fluorescence (25). We first confirmed that Cu(II) induced a reduction in tyrosine fluorescence of Aβ(1–42) at 307 nm (Fig. 1B), indicating that there is an interaction between Cu(II) and Aβ. When coincubated with cyc-KLVFF, Aβ(1–42) in the presence of Cu(II) exhibited greater fluorescence (Fig. 1B), indicating that the cyc-KLVFF may have removed the Cu(II) from the Aβ. To confirm this capture mechanism, the Cu(II) was incubated with Aβ first, fluorescence was measured, whereupon cyc-KLVFF was added to the sample, and fluorescence was reassayed after 1 h. The addition of cyc-KLVFF completely reversed the quenching of tyrosine fluorescence by Cu(II) (Fig. 1C). We also used the generation of H2O2 by Aβ-Cu(II) complexes as an index of copper interaction...
Cyc-KLVFF (0.5 mol eq) inhibited the amount of H₂O₂ generated by Cu(II)-Aβ(1–42) by 70% (Fig. 1D). Taken together, the data demonstrate that cyc-KLVFF can competitively capture Cu(II) from Cu(II)-Aβ.

Cyc(Cu(II))-KLVFF Inhibits and Reverses Aβ Aggregation—The cyc(Cu(II))-KLVFF molecule contains two parts, the Aβ recognition domain KLVFF and the lytic domain cyc(Cu(II)). To investigate whether the two parts of the molecule are necessary for the inhibition of Aβ aggregation, we used ThT fluorescence to measure the rate of amyloid formation over 7 days in the presence of KLVFF alone, cyc(Cu(II)) alone, cyc(Cu(II))-KLVFF mixed with KLVFF peptide, cyc-KLVFF (apo-hybrid), or cyc(Cu(II))-KLVFF (Fig. 2A). Incubation with cyc(Cu(II)), KLVFF + cyc(Cu(II)) mixture, or cyc-Cu(II)-KLVFF did not interfere with Aβ aggregation. Compared with KLVFF (~20% inhibition, as expected (19)), cyc(Cu(II))-KLVFF markedly inhibited the formation of ThT-positive aggregates (~90% inhibition, p < 0.01). These results indicate that connecting the lytic and the recognition components potentiate the inhibition of Aβ aggregation. TEM (19) confirmed that Aβ fibril formation was blocked by cyc(Cu(II))-KLVFF (Fig. 2B).

Cyc(Cu(II))-KLVFF Degrades Preformed Fibrillar Aβ(1–42)—Preformed fibrils of Aβ(1–42) (FAB(1–42)) were incubated with different concentrations of cyc(Cu(II))-KLVFF for 8 days, and the levels of residual ThT binding were assayed (Fig. 2C). The data indicate that cyc(Cu(II))-KLVFF reduced ThT binding by 60–75% in a dose-dependent manner. The disaggregation of FAB(1–42) was confirmed by TEM (Fig. 2D).

Cyc(Cu(II))-KLVFF Cleaves Fresh and Oligomerized Aβ into Fragments—We used Western blotting and mass spectrometry analyses to appraise the ability of cyc(Cu(II))-KLVFF to cleave Aβ species. Blots of freshly prepared Aβ (10 μM) incubated for 5 days with cyc(Cu(II))-KLVFF (40 μM) showed a loss of immunoreactivity consistent with a loss of the peptide (Fig. 3A). More Aβ(1–40) was lost than Aβ(1–42) under these conditions, suggesting that Aβ(1–42) is more resistant to degradation (Fig. 3A). Evidence supports a role for Aβ oligomers in the pathology of AD (27, 28). Therefore, we analyzed the interaction of cyc(Cu(II))-KLVFF with oligomeric Aβ by Western blotting. Cyc(Cu(II))-KLVFF was incubated with prepared Aβ(1–42) (5 μM) for 5 days. The cyc(Cu(II))-KLVFF appeared to degrade oAβ in a dose-dependent manner (Fig. 3B).

MALDI-TOF/MS analysis confirmed that incubation of freshly prepared Aβ(1–42) with cyc(Cu(II))-KLVFF for 5–7 days resulted in generation of Aβ fragments (Fig. 3C and supplemental Table S1). The cleavage sites were within 5 residues of the KLVFF motif (Fig. 3D). These data indicate that the
appearance of A\textsubscript{\beta} fragments and the disappearance of bands on Western blot are correlated and establish that cyc(Cu(II))-KLVFF degrades the A\textsubscript{\beta} peptide.

Cyc-KLVFF Protects Neurons from A\textsubscript{\beta} Toxicity—We reasoned that cyc(Cu(II))-KLVFF may protect neurons from A\textsubscript{\beta} toxicity, if it is itself nontoxic. N2a neuronal cells were untreated or pretreated with 10\,\mu{M} Cu(II) for 24 h, and then the culture medium was exchanged, whereupon the cells were incubated for 2 days with medium containing A\textsubscript{\beta}(1–42) alone (5\,\mu{M}) or A\textsubscript{\beta}(1–42) with apocyc-KLVFF. After incubation, neuronal viability was assessed by MTT assay, which revealed that A\textsubscript{\beta}(1–42) was toxic, and toxicity was exaggerated by Cu(II) pretreatment (78\% survival at 48 h) (Fig. 4A), in agreement with previous findings (11, 26). Apocyc-KLVFF rescued A\textsubscript{\beta}(1–42)-mediated toxicity even at concentrations (1\,\mu{M}) one-fifth those of the A\textsubscript{\beta}(1–42) in the culture (5\,\mu{M}; Fig. 4A). Apocyc-KLVFF concentrations 20-fold greater than the concentration needed to effect maximum rescue, there was no evidence of toxicity (data not shown), indicating that the cyc-KLVFF was tolerated by the primary neurons.

In similar experiments, we also appraised neuronal apoptosis as measured by TUNEL assay, and cell morphology was assessed by tubulin immunohistochemistry. As expected, treatment with A\textsubscript{\beta}(1–42) induced cell apoptosis as well as abnormal cell morphology: neurites became dystrophic, and some neurons detached from the slides (Fig. 4B). Coincubation of A\textsubscript{\beta}(1–42) with cyc(Cu(II))-KLVFF significantly reduced the number of TUNEL-positive cells (Fig. 4C) and prevented changes in cell morphology (Fig. 4B).

Cyclen Conjugation to Other A\textsubscript{\beta} Recognition Motifs—To explore the minimum and optimum recognition motif we replaced KLVFF with LVFF in the cyclen conjugate (cyc-LVFF; Fig. 5A). Meanwhile, glycine, a small and flexible amino acid, was introduced into the recognition domain (cyc-GKLVFF; Fig. 5A) to test any requirements for flexibility between the recog-
Cu-Cyclen Cleavage of Aβ

**A**

| (Cyc(Cu(II))-LVFF) | (Cyc(Cu(II))-GKL.VFF) |
|---------------------|------------------------|

**B**

![Graph showing the percentage of aggregation (%)](image)

**C**

![Graph showing the concentration of cleavage agents (μM)](image)

**FIGURE 5. Comparison of cyclen-hybrid Aβ cleavage agents.** A, chemical structures of the four alternatives to cyc(Cu(II))-KLVFF tested. B, effects of cyclen-liganded Aβ recognition agents (40 μM) on Aβ(1–42):Cu(II) (10 μM)–mediated H₂O₂ generation. Data are means ± S.D., n = 3. C, effects of cyclen-liganded Aβ recognition agents on Aβ(1–42) aggregation monitored by ThT fluorescence. Substances (0–160 μM) were added Aβ(1–42) (10 μM) and then coincubated for 3 days. The calculated average IC₅₀ is shown in the inset. Data are means ± S.D., n = 3. *p < 0.01.

**DISCUSSION**

The clearance or degradation of Aβ holds promise as a therapeutic approach to AD. The current data explore the potential of small molecules such as cyclen to act as lytic agents in living systems. Such potent hydrolytic activities may be expected to be harmful, but our current data show for the first time that Cu(II)-cyclen conjugates are tolerated in neuronal cell culture for periods of days at concentrations (20 μM) that may be expected to be in a therapeutic range. The conjugation of cyclen to an Aβ recognition moiety may have improved the tolerability of the lytic molecule to cells.

Cyclen relies upon binding redox-active metal ions (e.g., Co³⁺, Cu²⁺) for its lytic activity. Our data also show that apocyclen conjugates can capture Cu²⁺ that is bound to Aβ (Fig. 1B), inhibiting aggregation (Fig. 2A), H₂O₂ formation (Fig. 1C), and toxicity (Fig. 4A). This is important because ionic cobalt does not exist in biochemical systems (cobalt is not ionized in cobalamin), whereas ionic copper is enriched in several protein aggregates, such as amyloid. Furthermore, bound metal ions may not survive the digestive system or pass across the blood-brain barrier in oral or parenteral preparations, respectively. The apo-preparation also ensures that the cyclen complex remains inactive until it reaches its peptide target, such as Cu-bound Aβ. Of all biological metal ions, Cu²⁺ has by far the highest binding affinity to cyclen (Kₐ = 10⁻²³), more than 20 orders of magnitude greater than its affinity for Mg²⁺ or Ca²⁺, by comparison (29). Therefore, even if the cyclen ring becomes occupied by another metal ion en route to its peptide target (e.g., in the blood), the ability of apocyclen-KLVFF to rescue Aβ in the mixed metal ion environment of culture medium (Fig. 4A) suggests that ultimately enough Cu²⁺ exchanges into the cyclen ring at the peptide docking site to activate the lytic activity.

The data from MALDI-TOF/MS (Fig. 3C) show that the major cleavage sites locate to either sides of the KLFF, which suggests that the recognition domain of cyc-KLVFF is necessary for the process of cleaving Aβ. Cyc-KLVFF was well toler-
Aβ-Cu electrochemistry (38, 39). Several reports have confirmed that Aβ-Cu complexes are increased in AD brain (11, 13, 40), and plaques can be dissolved by copper chelators (41). Indeed, drugs that target Aβ-Cu interaction show therapeutic efficacy in preclinical AD models and early phase clinical trials (16, 42–46).

To our knowledge, this is first report of the use of a synthetic activity to rescue protein aggregate-mediated cellular toxicity. The principle of targeting pathological protein aggregates with a small recognition domain ligated to cyclen could have broad applicability to the many forms of amyloidosis and other diseases associated with protein aggregation, including several neurodegenerative diseases such as Huntington disease. Peripheral amyloidosis, like that formed by β2-microglobulin, might be even more readily accessible by the cyclen complexes than brain amyloids, especially as there is no blood-brain barrier to contend with. Given the relatively slow rate of lysis, high plasma levels may be needed for pharmacotherapy, and therefore this class of drug may be best delivered intravenously. Formal toxicology of an intravenous formulation will determine whether a therapeutic dose of these compounds can be tolerated. To maximize the chances of tolerability, we developed an apocyclen strategy that capitalizes on the peptide’s abnormal decoration with ionized copper. Other instances where protein aggregates are induced or decorated by copper include β2-microglobulin (47) and transthyretin (48). These might also be accessible targets for apocyclen ligands, especially as there is no blood-brain barrier to contend with. Given the relatively slow rate of lysis, high plasma levels may be needed for pharmacotherapy, and therefore this class of drug may be best delivered intravenously. Formal toxicology of an intravenous formulation will determine whether a therapeutic dose of these compounds can be tolerated. To maximize the chances of tolerability, we developed an apocyclen strategy that capitalizes on the peptide’s abnormal decoration with ionized copper. Other instances where protein aggregates are induced or decorated by copper include β2-microglobulin (47) and transthyretin (48). These might also be accessible targets for apocyclen ligands, and warrant further investigation.

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