TMPyP Inhibits Amyloid-β Aggregation and Alleviates Amyloid-Induced Cytotoxicity

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INTRODUCTION

Alzheimer’s disease (AD) is one of the common neurodegenerative disorders characterized by the accumulation of amyloid-β (Aβ) in brain. Aβ is composed of 39-42 amino acids and produced by the cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. The aggregation of Aβ leads to the formation of both oligomers and fibrils. Molecular dynamic simulations provide an insight into the interaction between TMPyP and Aβ at the molecular level. The half-maximal inhibitory concentrations of TMPyP acting on the oligomers and fibrils were determined to be 0.6 and 0.43 μM, respectively. As a member of porphyrin family, TMPyP is of rather low cytotoxicity, and the cytotoxicity of the Aβ aggregates was also relieved upon coincubation with TMPyP. The excellent performance of TMPyP thus makes it a potential drug candidate for AD therapy.
not easily absorbed by human bodies. To develop water-soluble drug candidates with good biocompatibility and low cytotoxicity and to further unravel the interaction mechanism between porphyrins and Aβ, the effect of 5,10,15,20-tetrakis(N-methyl-4-pyridyl)porphyrin (TMPyP), a planar and water-soluble cationic porphyrin, on the aggregation properties of Aβ was examined in detail.

Surface plasmon resonance (SPR) is highly sensitive to the tiny changes in the refractive index or thickness associated with a biomolecular interaction.24 By immobilizing the capture antibodies that are specific to Aβ oligomers and fibrils in separate fluidic channels, an SPR assay for real-time monitoring of Aβ aggregation was proposed by our group.25 In this study, the inhibition of Aβ aggregation and dismantling of the preformed Aβ aggregates by TMPyP were investigated by SPR and other methods. The possible binding sites of TMPyP to Aβ were determined by molecular dynamic simulations at the molecular level.

RESULTS AND DISCUSSION

TMPyP Inhibits Aβ Aggregation and Dismantles the Aggregates. Amyloid aggregation and the inhibition effect of TMPyP were first monitored by thioflavin-T (ThT) fluorescence assay7 (Figure 1A). As shown by the black curve, the fluorescence intensity of ThT upon incubation with 6.2 μM Aβ increased with the incubation time, and a plateau was reached beyond 48 h. When 6.2 μM TMPyP was coincubated with 6.2 μM Aβ, a much lower fluorescence intensity of ThT was attained (red curve), indicating that TMPyP interfered with ThT binding to amyloid fibrils. Similar trend was obtained in the case of TMPyP alone (blue curve). As can be seen by the magenta curve, the incubation of TMPyP with the preaggregated TMPyP (2 h) leads to a lower fluorescence intensity in comparison with that of Aβ alone (black curve), indicating that TMPyP was capable of dismantling the preformed fibrils. The ThT fluorescence assay is useful for monitoring the progression of fibril formation; however, it is not an effective tool for the detection of soluble and neurotoxic oligomers. Time-dependent SPR sensorgrams at the sensor chips preimmobilized with A11 antibody (Figure 1B) and OC antibody (Figure 1C) were acquired upon injection of the incubated samples. The A11 and OC antibodies were capable of recognizing the oligomers and fibrils of Aβ, respectively.25-27 The Aβ samples with (red curves) and without (black curves) TMPyP were serially flowed over the two channels and the amount of the bound species was measured by examining the difference in the baseline SPR angles before and after the injection. The nonspecific adsorption (2–3 mDeg) was subtracted upon injection of the incubated samples onto the 11-mercaptoundecanoic acid (MUA)-covered sensor chips. In the absence of TMPyP, the SPR signals of 20 mDeg in curve a of Figure 1B and 15 mDeg in curve a of Figure 1C were attained, indicating that the Aβ samples are monomer-dominated at the time point of 0 h,
although some oligomers and fibrils were preformed during the storage. Upon incubation of the Aβ samples alone for 6 h, the significantly increased SPR signals in both channels suggest the formation of well-ordered oligomers and fibrils (curve b in Figure 1B,C). Interestingly, the incubation of Aβ with TMPyP for 6 h remarkably decreased the SPR signals (curve c in Figure 1B,C), suggesting that TMPyP could inhibit Aβ oligomerization and fibrillation. Such an inhibition process could be interpreted as follows: TMPyP inhibits the formation of Aβ oligomers, and the lowered levels of the oligomers prevent the fibril formation. Furthermore, we noticed that upon incubation of the preaggregated Aβ (6 h) with TMPyP for 2 h, the smaller SPR signals in curve d than those in curve b of Figure 1B,C indicate that TMPyP could dismantle the mature aggregates of Aβ. Taken together, TMPyP could not only inhibit Aβ aggregation but also disrupt the preformed aggregates.

**Influence of TMPyP on the Secondary Structure of Aβ.**

Next, we assessed the conformational change of Aβ in the absence (Figure 2A) and presence (Figure 2B) of TMPyP by circular dichroism (CD) spectroscopy. At 0 h, Aβ exhibits natively unstructured conformation, as evidenced by the characteristic peak at 197 nm (curve a in Figure 2A). After incubation for 3 h, the disappearance of the 197 nm peak is accompanied by the appearance of a new negative peak at 215 nm (curve b in Figure 2A), which indicates the formation of β-sheet-structured oligomers. However, in the presence of TMPyP, Aβ reserves the unstructured conformation at 0 h (curve a in Figure 2B), and after incubation with TMPyP for 3 h, the negative peak at 215 nm disappears (curve b in Figure 2B). Most dramatically, upon incubation of the preincubated Aβ (3 h) with TMPyP for 2 h, less β-sheet aggregates with much attenuated negative peak at 215 nm were produced (curve c in Figure 2B), suggesting that TMPyP could slow
down the formation of the β-sheet structures. Thus, TMPyP serves as a potent inhibitor of the β-sheet formation (i.e., dimerization and oligomerization), as characterized by CD and SPR results. The contention was also supported by UV−vis spectroscopy (Figure 2C). Aβ does not show any absorbance peak over the wavelength range examined (curve a). The incorporation of 10 μM Aβ into 10 μM TMPyP shifted the absorbance peak of TMPyP from 422 to 429 nm (curves b and c), suggesting the π−π interactions between the porphyrin ring and Aβ.28 It has been documented that the porphyrin ring plays an important role in the prevention of Aβ aggregation by heme.22,28

Morphology of the Aβ Aggregates with and without TMPyP. The Aβ aggregates with and without TMPyP were clearly resolved from atomic force microscopy (AFM) imaging (Figure 3). Monomeric Aβ is soluble, and no aggregates were formed (Figure 3A). After 12 h incubation, spherical particles with diameters of about 2.5 nm were observed (Figure 3B). With the increase in the incubation time to 36 h, long, mature fibrils of Aβ with the nominal height of 5−6 nm were attained (Figure 3C). However, the morphology of the Aβ aggregates in the presence of TMPyP is totally different from that of Aβ alone. Few spherical particles were formed via incubating Aβ with TMPyP at a concentration ratio of 1:1 for 12 h (Figure 3E). With the elapse of the incubation time to 36 h, the spherical particles were still resolved and no fibrils were obtained (Figure 3F). It is clear that the incorporation of TMPyP prevents the Aβ monomers from further growing into oligomers or fibrils, consistent with our aforementioned experimental results. Note that the incubation time for AFM imaging is much longer than that for SPR and CD characterizations. The conversion of the unstructured Aβ to β-sheet containing structures, such as dimers, is a quick process, and these small Aβ oligomers could be recognized by the antibodies preimmobilized on the SPR chips. Because AFM only images well-formed oligomers and fibrils, the small Aβ oligomers could not be detected by AFM.

IC50 of TMPyP Inhibitor. The inhibition assay of TMPyP on the formation of Aβ oligomers (A) and fibrils (B) was conducted (Figure 4). The SPR signals as a function of TMPyP concentrations were measured and the concentration of TMPyP that causes 50% inhibition (IC50) was deduced. The IC50 values of 0.6 and 0.43 μM for TMPyP acting on the oligomers and fibrils, respectively, were attained, being lower than those of the porphyrin derivatives (3.56 and 11.0 μM)29 and other inhibitors, such as hematoxylin (1.6 μM)30 and brazilin (2.3 μM)30. It is worth noting that TMPyP suppresses the formation of both oligomers and fibrils, serving as a novel bifunctional inhibitor of Aβ aggregation. Fe-TMPyP possesses similar but weaker inhibition effect, which indicates that the metal ion center of porphyrins may not play an important role in inhibiting Aβ aggregation. However, several reports indicate that the metal ions, such as Zn2+ and Cu2+, play an important role in AD pathogenesis;31−33 thus, chelating metal ions is another way for the prevention of Aβ aggregation and the cure of AD.

**TMPyP Alleviates Aβ-Induced Cytotoxicity.** The cell cytotoxicity of Aβ in the absence and presence of TMPyP was assessed by cell counting kit-8 (CCK-8) assay34 (Figure 5).

![Figure 4](image-url) Dependence of the SPR responses on the concentrations of TMPyP. Ten micromolar concentration of Aβ was incubated with various concentrations of TMPyP for 6 h and the above solutions were flowed over the SPR chips precovered with A11 antibody (A) and OC antibody (B).

Human neuroblastoma SH-SYSY cells were incubated with Aβ, TMPyP, and the mixture of Aβ with different concentrations of TMPyP for 24 h and the survival rates were determined. It was found that only 71% of the cells remained viable when exposed to 20 μM Aβ for 24 h (lane 2), indicating the cytotoxicity of the Aβ aggregates. However, due to the inhibition effect of TMPyP on Aβ aggregation, reduced cytotoxicity (97%) was obtained when the cells were treated with the mixture of 20 μM Aβ and 20 μM TMPyP (lane 3). The decrease in the concentrations of TMPyP leads to a lowered cell viability (lanes 4−6), and the survival rate of the cells in the case of 20 μM Aβ and 2 μM TMPyP (lane 6) is similar with that in the presence of Aβ alone (lane 2). TMPyP possesses low toxicity and the
survival rate is approximately 94% (lane 7). The cell viability assay was consistent with the SPR results in that TMPyP inhibits Aβ aggregation in a dose-dependent manner.

**Binding Modes of TMPyP to Aβ.** To gain a better understanding of the inhibition mechanism, we performed all-atom molecular dynamic simulations to study the binding modes of TMPyP to the Aβ pentamer (Figure 6). It can be clearly seen that TMPyP does not disturb the structural integrity of the Aβ pentamer within 30 ns. TMPyP preferentially binds to the N-terminus and the salt-bridge region of Aβ. These results suggest that the attachment of TMPyP to the Aβ pentamer hinders the elongation and lateral aggregation of the pentamer.

**Possible Binding Sites of TMPyP to Aβ.** The inhibition mechanism and the possible binding sites were further demonstrated by molecular dynamic simulations (Figure 7). The Aβ pentamer–TMPyP complex was clustered into different structural groups using a root-mean-square deviation of 10 Å as the cutoff value, and highly populated binding sites at Ser8-His13 (region 1, 31.6%), Phe4-Ser8 (region 2, 10.6%), Asn27-Ile31 (region 3, 7.4%), Ala30-Leu34 (region 4, 6.2%), Ser8-Val12 (region 5, 6.2%), and Val39-Ala42 (region 6, 5.8%) were attained. TMPyP preferentially binds to the aromatic residues of Aβ, such as Phe4, Phe6, and Tyr10 through π–π interactions (regions 1, 2, and 5), which interferes with the ordered stacking of the β-sheets for the formation of large oligomers and fibrils.35,36 Because the hydrophobic region at the C-terminus of Aβ is capable of initiating the self-assembly of Aβ,37 the binding of TMPyP to the hydrophobic pocket (regions 3, 4, and 6) thus interferes with its aggregation (consistent with our AFM and SPR results). Furthermore, TMPyP binds to Asn27-Ile31 of Aβ (region 3), disrupting the β-sheet structure stabilized by the salt-bridge region (Asp23-Lys28) (supported by our CD data).38,39 Taken together, the insertion of TMPyP into the hydrophobic and salt-bridge regions not only inhibits the self-aggregation of Aβ but also disrupts the β-sheet formation. As a result, the elongation of the Aβ aggregates is largely prevented.

**CONCLUSIONS**

Via the specific recognition of the preimmobilized A11 and OC antibodies to the oligomers and fibrils, respectively, an SPR assay of the inhibition of Aβ aggregation by TMPyP has been proposed. TMPyP not only inhibits Aβ aggregation but also disassembles the preformed Aβ aggregates. The SPR results were further confirmed by CD, AFM, and molecular dynamic simulation characterizations. The inhibition effect might be ascribed to the π–π interactions between Aβ and TMPyP and the insertion of TMPyP into the hydrophobic and salt-bridge regions of Aβ, which interferes its aggregation and disrupts the β-sheet structure stabilized by the salt-bridge region. The IC50 values for TMPyP acting on the oligomers and fibrils were estimated to be 0.6 and 0.43 μM, respectively, being lower than those of the porphyrin derivatives and other inhibitors, such as hematoxylin and brazilin. As a member of porphyrin family, TMPyP possesses low cytotoxicity, and the cytotoxicity of the Aβ aggregates was relieved upon the incorporation of TMPyP. Thus, TMPyP not only inhibits Aβ aggregation but also alleviates amyloid-induced cytotoxicity, providing a new insight for the development of porphyrin-based therapeutic drugs for neurodegenerative diseases.
EXPERIMENTAL SECTION

Chemicals and Reagents. MUA, ethanolamine (EA), K2HPO4, KH2PO4, NaOH, N-(3-dimethylaminopropyl)-N'-(ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and TMPyP were acquired from Sigma-Aldrich (St. Louis, MO). Aβ(1−42) was obtained from Bachem (Torrance, CA). Monoclonal antibodies against oligomers and fibrils of Aβ (A11 and OC, respectively) were obtained from Millipore Inc. (Dedham, MA). All of the reagents were of analytical grade and used without further purification. Unless otherwise stated, all of the stock solutions were prepared daily with deionized water treated with a water purification system (Simplicity185; Millipore Corp., Billerica, MA).

Solution Preparation. To effectively inhibit the aggregation of Aβ(1−42), lyophilized Aβ(1−42) samples were dissolved in 10 mM NaOH solution. Upon sonication for 1 min, the solution was centrifuged at 13,000 rpm for 30 min to remove any insoluble particles, and the supernatants were pipetted out and diluted by phosphate-buffered saline (PBS, 100 mM phosphate, pH 7.4). Aβ(1−42) or the mixture of Aβ(1−42) and TMPyP was incubated at 37 °C for different time periods before assay. OC and A11 antibodies were prepared with PBS buffer and their concentrations were maintained at 2 nM. TMPyP was dissolved in 20 mM NaOH solution. MUA and EA were dissolved in ethyl alcohol and water, respectively. EDC/NHS solution was prepared by mixing 0.4 M EDC and 0.1 M NHS in water before the activation of MUA self-assembled monolayers (SAMs).

SPR Detection. SPR assay was performed on a BI-3000 system (Biosensing Instrument Inc, Tempe, AZ). Au films coated onto BK7 glass slides were annealed in a hydrogen atmosphere containing 5% (v/v) CO2 at 37 °C. The same threshold value was used for optimal binding sites. The Aβ pentamer−TMPyP complex was then immersed in a cuboid box filled with TIP3P water molecules, leaving at least a 12 Å buffering zone away from any boundary of the box. Each system was neutralized by adding Cl− counter ions.

Molecular Dynamic System Construction. A complete structure of Aβ pentamer was modeled to probe the TMPyP binding sites and modes. Briefly, the initial monomer coordinates of an Aβ(17−42) peptide were derived from the NMR structure (protein data bank code 2BEG) and then the unresolved N-terminus (residues 1−16) was constructed and reassembled to Aβ(17−42) to yield a full-length Aβ(1−42) monomer with the β hairpin structure. The whole structure of Aβ monomer consists of two antiparallel β-strands (residues Val1-Ser26 and Ile31-Ala42) linked by a U-bend (residues Asn27 and Ala50). The Aβ pentamer was constructed manually by stacking five monomers in parallel and registered form according to the NMR structure. To eliminate the potential collisions and gain a more reasonable initial structure, the oligomer was subjected to a molecular mechanics optimization. Five TMPyP molecules were initially and randomly placed around the relaxed Aβ pentamer with a minimal distance of 10 Å to allow them to land on optimal binding sites. The Aβ pentamer−TMPyP complex was then immersed in a cuboid box filled with TIP3P water molecules, leaving at least a 12 Å buffering zone away from any boundary of the box. Each system was neutralized by adding Cl− counter ions.

Molecular Dynamic Simulation. Simulation of the Aβ pentamer−TMPyP complex containing explicit water molecules and counter ions was performed using the AmberTools14 program (sander module) with the Amber ff14SB force field for protein and generalized AMBER force field for ligands. Restrained electrostatic potential charges for TMPyP were obtained with the PyRED server using Gaussian 09 (rev. D.01). The simulation begins with a three-phase energy minimization. In the beginning, 10 kcal/mol Å2 elastic constant was used to constrain the heavy atoms of the solute for 2000 cycles, followed by another 2000 cycles with the same position constraints on the backbone atoms of Aβ; finally, the whole system was relaxed without any constrain for 4000 cycles. After energy minimization, the system was gradually heated from 50 to 310 K in 50 ps and equilibrated at 310 K for 150 ps to adjust the size and density. Finally, 30 ns production molecular dynamic runs were conducted to examine the mutual dynamics and binding events between Aβ and TMPyP. A time step of 2 fs was used to integrate the equations of motion, permitted by constraining the fast stretching of the hydrogen atoms covalently linked to the heavy atoms. The Coulomb potentials were handled by the smooth particle-mesh Ewald method with a direct space cutoff of 9.0 Å. The same threshold value was also used for the truncation of the Lennard–Jones potentials, whereas long-range analytic corrections were applied to the energy and pressure. Molecular dynamic trajectories were saved every 2 ps for subsequent analyses. All of the calculations were performed on the MolDesigner Molecular Simulation Platform.

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Y.F. and L.W. collected the SPR data and CD spectra. Y.X. and D.W. performed the UV–vis and AFM characterizations. Y.X. and Z.W. conducted the cytotoxicity experiments. Y.X. and J.W. directed the project, analyzed the results, and wrote the manuscript. H.T., Q.L., and Z.Z. gave some useful comments.

Notes
The authors declare no competing financial interest.

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