Drug Discovery

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Polyoxometalates as Inhibitors of the Aggregation of Amyloid β Peptides Associated with Alzheimer's Disease**

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Alzheimer's disease (AD) is the most common form of dementia. It is characterized by cerebral extracellular amyloid plaques and intracellular neurofibrillary tangles.[1] Although the molecular mechanisms of AD pathogenesis are not clearly understood owing to its complexity, recent advances have demonstrated that the polymerization of amyloid β-peptides (Aβ) into amyloid fibrils is crucial.[2] Therefore, the development of Aβ inhibitors has received much attention. Peptides or peptide mimetics[3–5] and small organic molecules[6,7] are now the two main classes of Aβ inhibitors.

Since the discovery of the antitumor activity of cisplatin, many inorganic compounds have been used as important therapeutic drugs and diagnostic imaging agents.[8] Worldwide sales of inorganic drugs are growing rapidly. However, few inorganic ligands have been reported that can inhibit Aβ amyloid formation.[9,10] As important inorganic drug candidates, polyoxometalates (POMs) have shown promising antiviral and antitumor activities for more than a decade.[11–16] On the basis of cell-culture assays, in vitro enzymatic activity, and molecular-modeling studies, POMs have regained considerable attention as a result of their remarkable effect against acquired immune deficiency syndrome (AIDS), as demonstrated by Hill, Pope, and coworkers.[12,13] POMs can specifically bind to the basic fibroblast growth factor (bFGF) and induce dramatic conformational changes in the growth factor.[16]

POMs are early-transition-metal–oxygen-anion clusters with incomparable structural versatility and interesting properties. The unique feature of POMs is that nearly every molecular property that can influence the recognition and reactivity of POMs with target biological macromolecules is changeable, including polarity, redox potential, surface charge distribution, shape, and acidity.[11d] Several research groups have reported detailed studies of the interaction between POMs and their target proteins.[12–16] Given that the size and fairly rigid, cagelike structure of many POMs are similar, and in some cases nearly identical, to the size and structure of the water-solubilized fullerene derivatives reported to have fairly good antiamyloid activity,[10] POMs may also be potential Aβ inhibitors.

By using a high-throughput screening method based on the fluorescence of an Aβ–enhanced cyan fluorescent protein (ECFP) fusion expression system constructed in our laboratory and originally developed by Hecht and co-workers,[17] we have identified four POMs capable of inhibiting Aβ aggregation. The highest inhibition was observed for $K_8[P_2CoW_{17}O_{61}]$ (see Figure S1 in the Supporting Information), a phosphotungstate with a Wells–Dawson structure (Figure 1 A). POMs with a Keggin structure (Figure 1 B) showed moderate to high inhibition of Aβ aggregation. Smaller POMs, such as $Na_5[IMo_6O_{24}]$ with an Anderson structure (Figure 1 C), were inactive.

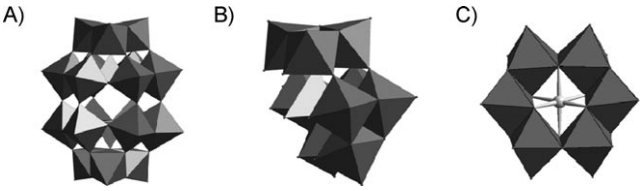

Figure 1. Structures of typical polyoxometalates: A) Wells–Dawson structure, $K_8[P_2CoW_{17}O_{61}]$; B) trivacant Keggin structure, α-$Na_9H[SiW_9O_{34}]$; C) Anderson structure, $Na_5[IMo_6O_{24}]$.

To verify the effect of POMs on the assembly of Aβ1–40 into amyloid fibrils, we used an in vitro thioflavin T (ThT) fluorescence assay. ThT, an extrinsic fluorescent dye, is able to bind to amyloid fibrils; upon binding, its fluorescence intensity increases.[18] Fibril formation was quantified by measuring the fluorescence intensity of ThT at 480 nm upon excitation at 444 nm. When fresh Aβ1–40 alone was incubated at 37 °C, ThT fluorescence as a function of incubation time showed a sigmoidal shape (Figure 2 A). This result is consistent with the nucleation-dependent polymerization model. However, in the presence of $K_8[P_2CoW_{17}O_{61}]$ (10 μM or 100 μM), ThT fluorescence did not increase, which indicated that Aβ amyloid formation was suppressed, in agreement with our fluorescence cell-based screening results. In the control experiment, there was no POM to influence the fluorescence of ThT (see Figure S2). Furthermore, Aβ1–40 from the stock solution (without incubation) adopted the

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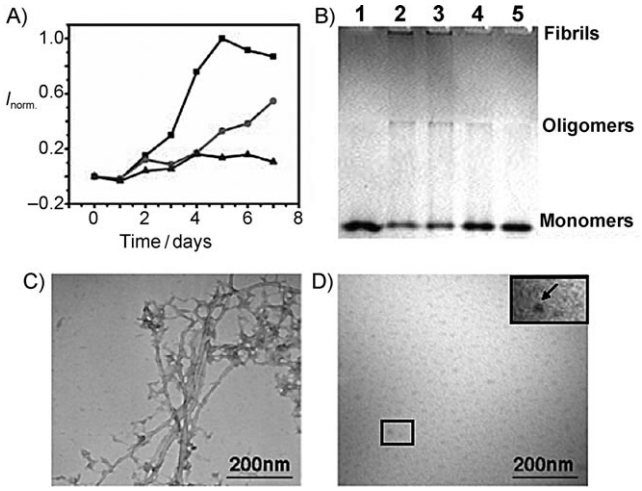

Figure 2. Inhibition of Aβ assembly by $K_8[P_2CoW_{17}O_{61}]$. A) Fibrillation kinetics of Aβ1–40 as monitored by the development of thioflavin T binding in the absence of $K_8[P_2CoW_{17}O_{61}]$ (■) or in the presence of $K_8[P_2CoW_{17}O_{61}]$ in a 5:1 (●) or 1:2 (▲) Aβ1–40/$K_8[P_2CoW_{17}O_{61}]$ ratio. B) Determination of the dose-dependent effect of $K_8[P_2CoW_{17}O_{61}]$ on the formation of Aβ fibrils by native PAGE. Samples were prepared as described in the Experimental Section with an increasing amount of $K_8[P_2CoW_{17}O_{61}]$ and without $K_8[P_2CoW_{17}O_{61}]$: 1) control (Aβ1–40 monomer), 2) Aβ1–40, 3) 50:1 Aβ1–40/$K_8[P_2CoW_{17}O_{61}]$, 4) 5:1 Aβ1–40/$K_8[P_2CoW_{17}O_{61}]$, 5) 1:2 Aβ1–40/$K_8[P_2CoW_{17}O_{61}]$. C) TEM micrograph of a control sample containing Aβ1–40 (50 μM) in 10 mM Tris buffer (pH 7.4) after incubation at 37 °C for 7 days. D) TEM micrograph of a sample containing Aβ1–40 (50 μM) and $K_8[P_2CoW_{17}O_{61}]$ (1:2 molar ratio) in 10 mM Tris buffer (pH 7.4) after incubation at 37 °C for 7 days. The inset is a high-magnification image of spherical Aβ1–40 oligomers.

monomeric form, as shown by native PAGE (Figure 2B, lane 1). After incubation for 7 days, the monomer band became weak, which suggested that Aβ1–40 had aggregated into higher-order oligomers and fibrils (Figure 2B, lane 2). Aβ amyloid formation was inhibited by $K_8[P_2CoW_{17}O_{61}]$, as shown by a stronger monomer band and weaker oligomer and fibril band in the native gel (Figure 2B, lanes 3–5).

We also investigated the effect of $K_8[P_2CoW_{17}O_{61}]$ on the morphology of Aβ aggregates by negative-stain TEM.[19] A 50 μM solution of Aβ was incubated with and without $K_8[P_2CoW_{17}O_{61}]$ in an amyloid-forming buffer (10 mM 2-amino-2-hydroxymethylpropane-1,3-diol (Tris), pH 7.4) at 37 °C for 7 days. Aβ formed long unbranched fibrils with diameters of about 10 nm: a typical structure for amyloid fibrils (Figure 2C). In contrast, in the presence of $K_8[P_2CoW_{17}O_{61}]$ (100 μM), spherical structures with heights of typically 3–4 nm were formed (Figure 2D). These results further support those found with ThT and indicate that $K_8[P_2CoW_{17}O_{61}]$ can inhibit Aβ-fibril formation.

To better quantify the inhibitory effect of POMs and to attempt to correlate this effect with structural or compositional features, we determined the IC_{50} value of a series of POMs in a ThT assay (see Figure S3). We studied a number of Dawson, Keggin, and Anderson structures to compare the effects of structure and charge (Table 1) and found that the inhibitory efficiency is related to the POM structure. The data obtained indicate that the inhibitory efficiency increases with

Table 1: Inhibitory efficiency of POMs with respect to Aβ fibrillization.

	Structure type	Charges	K_a[a] (M^{-1})	IC_{50}[b] (μM)
$Na_5[IMo_6O_{24}]$	Anderson	5	–	No inhibition
$K_7[PTi_2W_{10}O_{40}]$	Keggin	7	4.65×10^5	39.04
α-Na_9H-$[SiW_9O_{34}]$	Keggin	10	4.55×10^6	19.85
$K_8[\beta\text{-}SiW_{11}O_{39}]$	Keggin	8	4.76×10^5	39.02
$K_8[P_2CoW_{17}O_{61}]$	Dawson	8	2.08×10^6	16.68
$H_3[PMo_{12}O_{40}]$	Keggin	3	$<1\times10^5$	No inhibition

[a] Tyrosine-fluorescence titration data were used to estimate the binding constant by nonlinear least-squares fitting. [b] A ThT assay was used for determination of the IC_{50} value.

the size of the administered POM. The small Anderson compound studied was inactive, larger Keggin compounds were moderately active, and the largest, Dawson compound was the best inhibitor. These results indicate that the size of the POM might play a key role in Aβ recognition and amyloid inhibition.

The four POMs of the Keggin type showed different inhibition abilities. To gain a better understanding of the inhibition activity of the Keggin-type POMs, we compared the binding affinity of the four POMs for Aβ. The fluorescence intensity of Aβ was more strongly quenched as the amount of the different POMs increased. The apparent binding constants yielded by a nonlinear least-squares fit[20] (see Figure S4) were in agreement with the IC_{50} values of the POMs against Aβ aggregation (Table 1). Therefore, we concluded that the inhibition ability of a given type of POM depended mainly on the binding affinity for Aβ. As the binding affinities of the POMs were in agreement with their surface charges (Table 1), we assumed that electrostatic effects play an important role in the interaction between Aβ and POMs. This hypothesis is further supported by the inhibitory effect of $[NaP_5W_{30}O_{110}]^{14-}$ with a Preyssler structure, which shows a stronger inhibitory effect than that observed for the Dawson-type POM (Figure 3). These results further indicate that the inhibition selectivity of POMs might be related to size-specific electrostatic interactions between POMs and Aβ, although other interactions, such as hydrogen bonding, cannot be excluded.

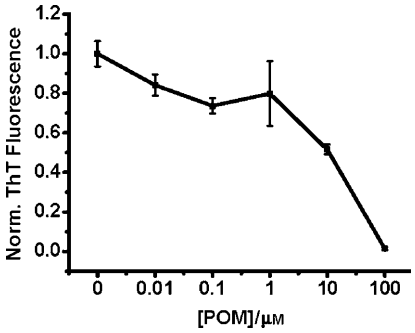

Figure 3. Dose-dependent inhibition of Aβ fibrillization by $[NaP_5W_{30}O_{110}]^{14-}$, which has a Preyssler structure. The excitation wavelength was 444 nm, and the emission intensity at 480 nm was used for analysis.

Next, we carried out digestion experiments with trypsin to investigate how POMs bind to Aβ. Trypsin digestion cleaves exclusively C-terminal residues up to the first arginine or lysine residue. The results (see Figure S5A) showed that Aβ was almost completely degraded after 60 min, whereas proteolysis was prevented in the presence of $K_8[P_2CoW_{17}O_{61}]$. This protective effect was dose-dependent, and complete inhibition was observed at the molar ratio POM/Aβ = 2:1. Under similar conditions, in the presence of $K_8[P_2CoW_{17}O_{61}]$, the trypsin-induced degradation of lysozyme was not affected (not shown). These results suggest that $K_8[P_2CoW_{17}O_{61}]$ can interact with the Aβ monomer and protect it from trypsin-induced degradation, and that the positively charged arginine or lysine residues of Aβ play an important role in the binding of $K_8[P_2CoW_{17}O_{61}]$ with Aβ. A salt effect on POM binding also showed that specific electrostatic interactions are important. With increasing ionic strength, the binding affinity[20] decreased dramatically, as reflected by an increase in the intensity of tyrosine fluorescence (see Figure S5B).

On the basis of the above results, we further investigated the specific POM-binding site on Aβ. Hill and co-workers demonstrated that POMs can bind to a cationic pocket of lysine residues on the outer surface of the flaps that cover the active site.[12] We reported previously that several classic POMs bind to the cationic pocket of basic fibroblast growth factor.[16] These observations may suggest that the cationic cluster HHQK of Aβ could be the binding site for POMs. We first used competition assays to verify the putative POM-binding site on Aβ. The cationic cluster of Aβ is near the central hydrophobic area of the previously described binding site for 4,4′-bis(1-anilinonaphthalene 8-sulfonate) (bis-ANS).[21,22] The fluorescence intensity of bis-ANS was strongly enhanced upon binding to Aβ. The addition of $K_8[P_2CoW_{17}O_{61}]$ to the Aβ–bis-ANS complex resulted in a progressive decrease in the intensity of bis-ANS fluorescence (see Figure S6). This result suggests that the binding of $K_8[P_2CoW_{17}O_{61}]$ to Aβ can influence the binding of bis-ANS to Aβ and implies that the binding site for POMs is near that for bis-ANS. Fluorescence-quenching data were fit with the Stern–Volmer equation,[23] which yielded a quenching constant of $0.88 \times 10^6\,\text{M}^{-1}$.

We also used the shorter fragment Aβ12–28 with the sequence VHHQKLVFFAEDVGSNK, which, like Aβ1–40, can bind to bis-ANS.[21] $K_8[P_2CoW_{17}O_{61}]$ showed competitive binding to Aβ12–28 with bis-ANS. The Stern–Volmer quenching constant was $0.90 \times 10^6\,\text{M}^{-1}$ (see Figure S7A), which is a similar value to that observed for Aβ1–40. To study the binding site further, we carried out a digestion experiment with trypsin. We chose Aβ12–28 as the trypsin substrate; the cleavage site was just next to the central hydrophobic region. It was clearly shown that $K_8[P_2CoW_{17}O_{61}]$ could prevent the digestion of this fragment (see Figure S7B), which indicates that $K_8[P_2CoW_{17}O_{61}]$ did bind this region of the Aβ peptide. Studies both in vivo and in vitro have suggested that low-molecular-weight (LMW) glycosaminoglycans (GAGs), such as LMW heparin or LMW chondroitin sulfate, might decrease the cytotoxic effect of Aβ and prevent the deposit of amyloid plaques through binding to Aβ to interfere with its fibril formation.[24] The binding site of heparin analogues to Aβ was speculated to be the region of His13–Lys16.[25] Our results clearly show that POMs bind exclusively to a positively charged recognition motif, presumably HHQK, that is located in the 12–28 sequence of Aβ.

Previous studies have shown that Aβ-recognition agents modified with bulky groups, such as steroids, can be used as a strategy to hinder further Aβ association.[26,27] We assumed that POMs could act as both recognition elements and agents to hinder Aβ fibrillization owing to their charged nature and bulky size. There are two possible mechanisms by which POMs could inhibit Aβ fibrillization. Our observations clearly showed that POMs could bind to the Aβ monomer. This binding would lower the concentration of the free monomer and shift the equilibrium away from fibrillization. Another possibility is that the interactions between the large POM surface and Aβ oligomers could result in unfavorable conditions for nucleation and fibril growth through the blocking of direct contact between monomers. This situation would lead to the depletion of sub- and near-critical oligomers and to partial blockage of the kinetic pathway until the surface is saturated and new nuclei can be grown.[28]

One issue that needs to be addressed is the stability[16] of the Keggin and Wells–Dawson anions in dilute aqueous solution. POM structures are known to depend on concentration, the pH value, and the buffer characteristics. We quantified the stability of these compounds experimentally by UV/Vis spectroscopy (see Figure S8). Under the most commonly used conditions for the experiments reported herein, all the POMs used remained intact.[29–31]

The ability of POMs to inhibit Aβ assembly suggested that they might be useful in blocking Aβ-mediated cellular toxicity. To address this question, we used PC12 cells in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to probe cellular metabolism. Aβ was incubated with POMs for 7 days, and then cells were exposed to the Aβ/POM mixtures. The results of the assays were normalized by using the results for cells not treated with Aβ fibrils as a positive control. In the presence of POMs, the survival of cells increased to about 90% at most (Figure 4A). POMs themselves were nontoxic at the same concentrations (Figure 4B). Thus, POMs are not only effective in vitro inhibitors, as shown by the ThT fluorescence assay, but their inhibition effects can be demonstrated in PC12 cells as well.

In summary, through the use of a high-throughput screening method based on the fluorescence of an Aβ–ECFP fusion expression system and a ThT fluorescence assay, we have shown that four kinds of POMs serve as Aβ inhibitors. An electrostatic effect is the main factor in the interactions between POMs and Aβ. The size of POMs might play a key role in Aβ recognition and amyloid inhibition. Experiments based on enzyme digestion, fluorescence competitive binding, fluorescence quenching, and the use of a different Aβ fragment indicated that POMs bind to the positively charged His13–Lys16 cluster region of Aβ. To the best of our knowledge, it has not been reported previously that polyoxometalates can inhibit amyloid-β aggregation. We expect that

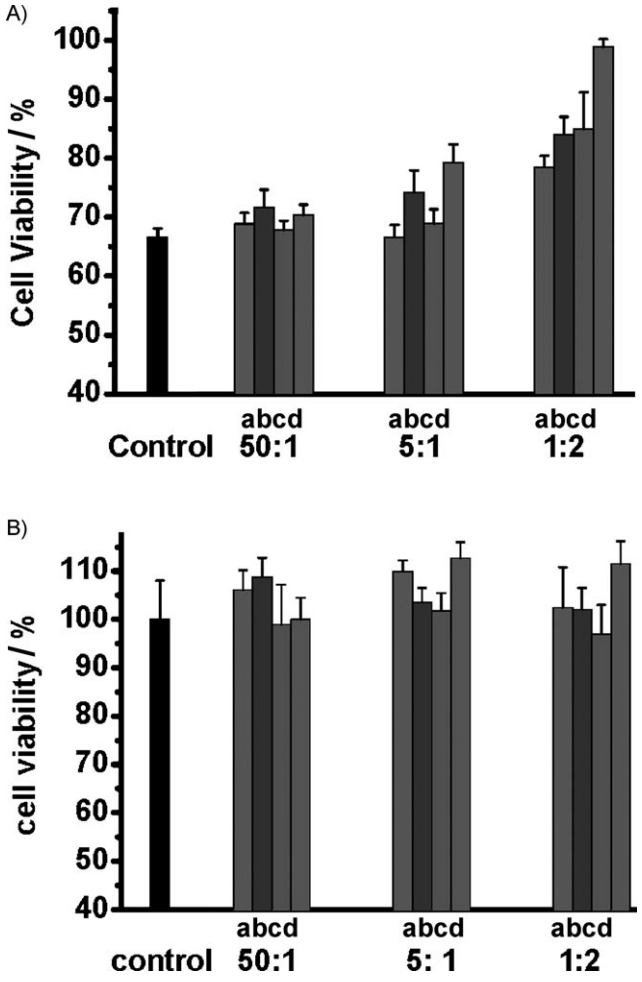

Figure 4. Effect of POMs ($K_7[PTi_2W_{10}O_{40}]$ (a); $K_8[\beta\text{-}SiW_{11}O_{39}]$ (b); $\alpha\text{-}Na_9H[SiW_9O_{34}]$ (c); $K_8[P_2CoW_{17}O_{61}]$ (d)) on the cell toxicity of Aβ. Samples were prepared according to the Experimental Section in the presence (A) or absence (B) of Aβ (5 μM). The cytotoxic effect on PC12 cells was determined by using an MTT assay. Aβ/POM ratios are given.

this study will prompt the design and screening of inorganic compounds as therapeutic agents for AD.

Experimental Section

High-throughout screening of Aβ inhibitors: The Aβ–ECFP fusion system was constructed as described with some modification.[17] Briefly, *Escherichia coli* strain BL21 (DE3) was transformed by the vector ALC (Aβ–linker–ECFP) or the control vector CFP (linker–ECFP) and cultured at 37°C in lysogeny broth (LB) containing ampicillin (50 $\mu g\,mL^{-1}$). Different POMs were added to the culture medium 30 min prior to protein expression induced by isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM). After expression of the recombinant proteins for 3 h, all samples were diluted to an optical density of 0.1 at 600 nm. The fluorescence of each sample was measured at 512 nm (excitation 433 nm) with a JASCO FP6500 spectrofluorometer. Compounds were tested in triplicate at a final concentration of 100 μM. The identification of hits was consistent across several repeated experiments.

Sample preparation: Aβ1–40 and Aβ12–28 were purchased individually from American Peptide (lot no. U10012) and Sigma (lot no. 32K12201). Peptides were prepared as previously described.[32–34] Briefly, the powdered Aβ peptide was first dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at a concentration of 1 $mg\,mL^{-1}$. The solution was shaken at 4°C for 2 h in a sealed vial for further dissolution and was then stored at −20°C as a stock solution. Before use, the solvent HFIP was removed by evaporation under a gentle stream of nitrogen, and the peptide was dissolved in 10 mM Tris buffer (pH 7.4). Fibril formation was carried out essentially as described earlier.[32–34]

ThT fluorescence spectroscopy: The kinetics of Aβ aggregation were monitored by using the dye ThT, the fluorescence of which is dependent on the formation of amyloid fibrils. Fluorescence measurements were carried out with a JASCO FP6500 spectrofluorometer. The fluorescence signal (excitation at 444 nm) was recorded between 460 and 650 nm; 10 nm slits were used for both emission and excitation measurements. The peptide concentration was 1 μM, and the ThT concentration was 10 μM. At different times, aliquots of the Aβ solution were taken for fluorescence measurements.

Native gel electrophoresis: Samples (10 μL) were analyzed by 20% native PAGE. Gels were run in a Tris/glycine system and developed by the silver-stain method.

Transmission electron microscopy: Samples (10 μL) were spotted onto carbon-coated copper grids and left for 30 min. The grids were then blotted with filter paper to remove excess buffer, and the sample was stained with 1.5% (w/v) phosphotungstic acid (pH 7.4). Grids were blotted again and air-dried before analysis on a transmission electron microscope (JEOL JEM-1011) operating at a voltage of 100 kV.

Trypsin digestion: Aβ (20 μM) was preincubated in 10 mM Tris buffer (pH 7.4) in the absence or presence of POM (in different Aβ/POM ratios) for 30 min at 37°C and then subjected to proteolysis by trypsin (0.1 $mg\,mL^{-1}$) under the same conditions for 10 min. At the end of the reaction, all samples were supplemented with SDS-PAGE reducing sample buffer, heated at 100°C for 5 min, and subjected to SDS-PAGE (20%). The gels were then silver-stained.

Fluorescence titration: The intrinsic tyrosine fluorescence was examined as previously described.[20] The concentration of Aβ1–40 was 3 μM. The excitation wavelength was 278 nm, and the emission intensity at 305 nm was used for analysis. The 1:1 binding stoichiometric equation was used to calculate the binding constants.

Bis-ANS displacement: Solutions of freshly prepared Aβ1–40 or Aβ12–28 with bis-ANS were incubated in 10 mM Tris buffer (pH 7.4) for 30 min at room temperature, and then bis-ANS fluorescence was measured. The POM was titrated into the solution every 10 min. The excitation wavelength was 395 nm.

Cell-toxicity assays: PC12 cells (rat pheochromocytoma, American Type Culture Collection) were cultured in Iscove modified Dulbecco medium (IMDM, Gibco BRL) supplemented with 5% fetal bovine serum and 10% horse serum in a humidified 5% CO_2 environment at 37°C. Cells were plated at a density of 10000 cells per well on 96-well plates in fresh medium (90 mL). After 24 h, Aβ1–40 peptides (5 μM) that had been aged with or without POMs were added, and the cells were further incubated for 48 h at 37°C. Cytotoxicity was measured by using a modified MTT assay kit (Promega). Absorbance values of formazan were determined at 490 nm with an automatic plate reader.

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