Amphiphilic Stilbene Derivatives Attenuate the Neurotoxicity of Soluble $A\beta_{42}$ Oligomers by Controlling Their Interactions with Cell Membranes

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Abstract

Misfolded proteins or polypeptides commonly observed in neurodegenerative diseases, including Alzheimer's disease (AD), are promising drug targets for developing therapeutic agents. To target the amyloid-β (Aβ) plaques and oligomers, the hallmarks of AD, we have developed twelve amphiphilic small molecules with different hydrophobic and hydrophilic fragments. *In vitro* binding experiments (i.e., fluorescence saturation assays) demonstrated that these amphiphilic compounds show high binding affinity to both Aβ plaques and oligomers, and six of them exhibit even higher binding affinity toward Aβ oligomers. These amphiphilic compounds can also label *ex vivo* Aβ species in the brain sections of transgenic AD mice, as shown by immunostaining with an Aβ antibody. Molecular docking studies were performed to help understand the structure-affinity relationships. To our delight, four amphiphilic compounds can alleviate Cu^{2+}-Aβ induced toxicity in

mouse neuroblastoma N2a via cell toxicity assays. In addition, confocal fluorescence imaging studies provided evidence that compounds ZY-15-MT and ZY-15-OMe can disrupt the interactions between Aβ oligomers and human neuroblastoma SH-SY5Y cell membranes. Overall, these studies suggest that developing compounds with amphiphilic properties that target Aβ oligomers can be an effective strategy for small molecule AD therapeutics.

Keywords

Amphiphilic molecules; amyloid-β (Aβ) oligomers; binding affinity; cell membranes; neurotoxicity; Alzheimer's Disease

Introduction

Misfolded proteins or polypeptides are commonly observed in different neurodegenerative diseases, including the aggregated amyloid-β (Aβ) peptides and tau proteins in Alzheimer's disease (AD) or the aggregated α-synuclein in Parkinson's disease.[1-3] AD being the most prevalent neurodegenerative disease, affects more than 44 million people worldwide, yet there is still a lack of effective treatment clinically.[4-5] Although insoluble Aβ fibrils have been considered the main hallmark of AD over the past century, it was not until recent decades that soluble Aβ oligomers were found to be the most neurotoxic species that directly affect synapse loss and neuronal injury.[6-9] Since Aβ oligomer species appear dominantly in the early stage of the disease and are the cause of continuous synaptic damage, they have become attractive targets for the drug development of AD.[10-11] Therefore, numerous strategies have been developed to target soluble Aβ oligomers from various antibodies,[12-13] polypeptides,[14-15] natural products,[16-20] as well as synthetic small molecules to inhibit or modulate their fibrilization process for the treatment of AD. Small molecules utilized as therapeutic agents for central nervous system disorders have advantages over biologics, including low molecular weight for higher blood-brain barrier (BBB) permeability and simpler structures for easy access at low cost.[21] However, due to the lack of molecular-level understanding of Aβ oligomers' structures, there is no effective way of designing small molecules with high Aβ oligomers binding affinity to modulate their neurotoxicity for AD drug development.

With the aim of developing therapeutic agents to alleviate Aβ oligomers' neurotoxicity on neuron cells, we herein reported an effective strategy by developing amphiphilic small molecules.

Twelve amphiphilic compounds with different amphiphilicity were developed by inking various hydrophobic stilbene derivatives with hydrophilic azamacrocycle (Me_2TACN) (Figure 1). The binding affinity of the amphiphilic compounds was measured by *in vitro* fluorescence saturation assays. These compounds show a high binding affinity to both amyloid fibrils and oligomers at a low micromolar range. More importantly, six of the compounds exhibit an even higher binding affinity toward Aβ oligomers. Molecular docking studies were also performed to understand the structure-affinity relationship for future design. The immunostaining confirmed the abilities of labeling *ex vivo* Aβ species of the compounds with an Aβ antibody HJ 3.4 in the brain sections of transgenic AD mice. Cell toxicity studies demonstrated that four compounds could rescue mouse neuroblastoma N2a cells from Cu^{2+}-Aβ induced toxicity. Finally, taking advantage of confocal microspore imaging technique, ZY-15-MT and ZY-15-OMe were found to decrease the interactions between Aβ oligomers and human neuroblastoma SH-SY5Y cells. These findings in the study provide lead compounds for future optimization and demonstrate an effective strategy for small molecular-based AD treatment.

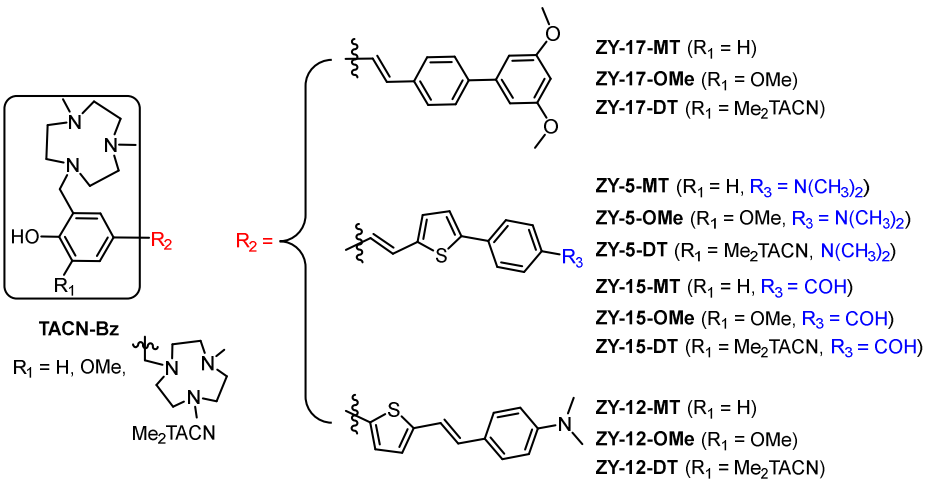


Figure 1. Molecular structure of the twelve amphiphilic compounds.

Results and discussion

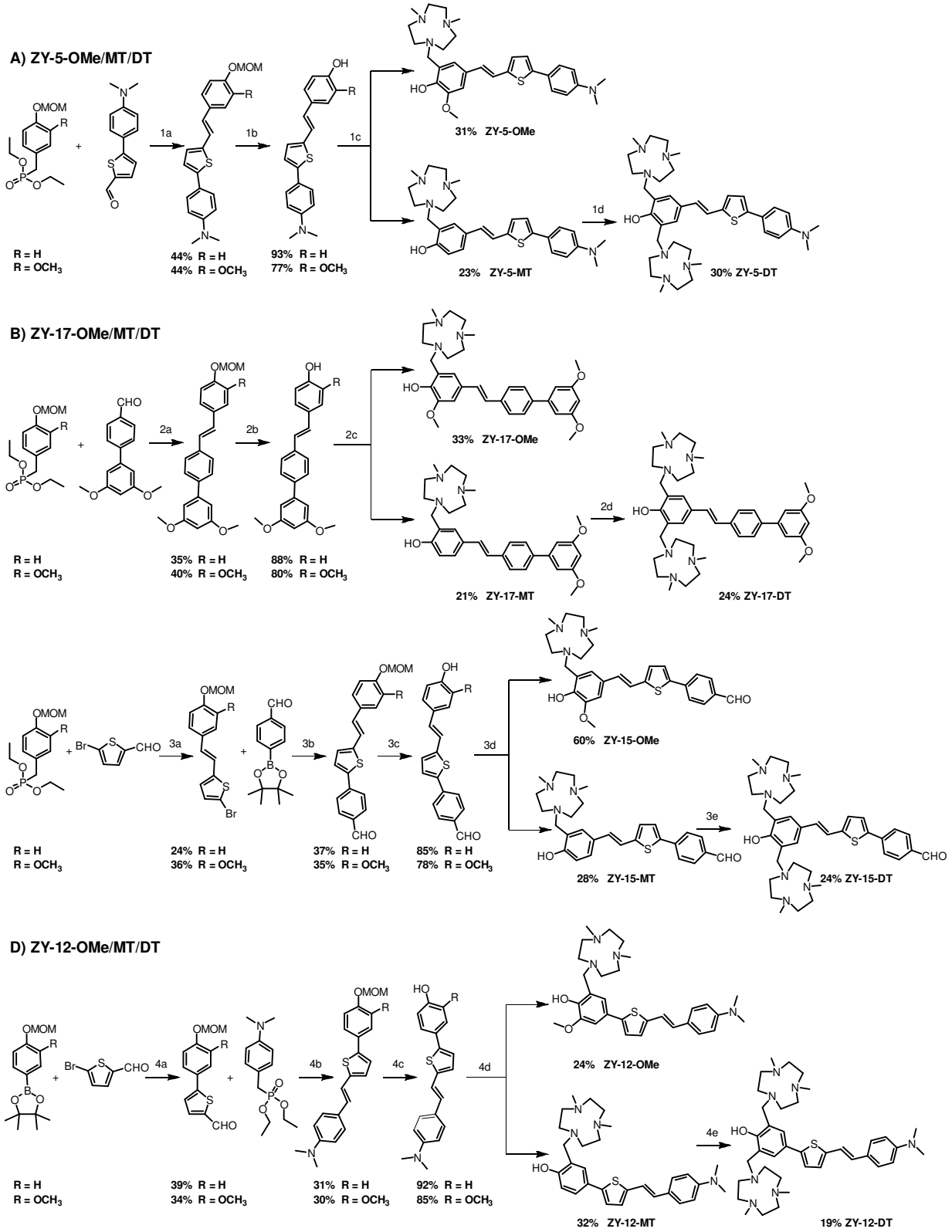
Design and synthesis of the amphiphilic compounds

Inspired by the amphiphilic nature of the Aβ peptide, we proposed that developing compounds with amphiphilic properties targeting Aβ species could effectively treat AD. Recently, peptidomimetic-based amphiphilic compounds were shown to inhibit Aβ fibrillation process and attenuate Aβ cytotoxicity in both neuroblastoma N2a and human neuroblastoma SH-SY5Y cells.[22]

Moreover, our group also successfully developed an amphiphilic small molecule, LS-4, that can serve as a therapeutic and imaging agent for Aβ oligomers in AD.[23] LS-4 was synthesized by attaching a hydrophilic azamacrocycle, 2,4-dimethyl-1,4,7-triazacyclononane (Me_2HTACN), to a hydrophobic distyryl stilbene derivative. Interestingly, because of the incorporation of the hydrophilic TACN fragment, the binding affinity of LS-4 toward both Aβ fibrils and oligomers increased dramatically comparing with Pre-LS-4, which only has the hydrophobic binding fragment. Although LS-4 showed very high binding affinity to Aβ fibrils (K_d = 58 ± 15 nM) and Aβ oligomers (K_d = 50 ± 9 nM), there is no high selectivity of LS-4 toward Aβ oligomers. Considering the higher neurotoxicity of Aβ oligomers and their appearance in the early stages of AD, it is beneficial to develop compounds with affinity and selectivity toward Aβ oligomers. For this purpose, we designed a series of compounds with different amphiphilicity by adding different hydrophobic aromatic ring systems and changing the number of hydrophilic ligands (TACNs) bound to the stilbene-derivatives. More specifically, we designed the TACN-Bz component of the compounds with 1) a hydroxyl group with one Me_2TACN (where R1 = H, giving ZY-#-MT series), 2) a methoxy group with one Me_2TACN (where R1 = OMe, giving ZY-#-OMe series), and 3) two Me_2TACN groups (where R1 = Me_2TACN, giving ZY-#-DT series). In the ZY-#-OMe series, the methoxy group ortho to the hydroxyl group was introduced as its interaction with Aβ oligomers was reported before.[24] For the R2 component of the molecules, we incorporated different aromatic ring systems (thiophene-benzene or benzene-benzene) with different substituents for their potential hydrophobic interactions (π-π interaction) with Aβ species. Based on this design approach, twelve compounds were designed for structure–activity relationships (SAR) studies in further analyses.

These 12 compounds were synthesized in similar synthetic pathways (Scheme 1). For ZY-5-OMe/MT/DT and ZY-17-OMe/MT/DT compounds (Scheme 1A and 1B), Horner-Wadsworth-Emmons (HWE) olefination reaction was used to form (E)-selective olefins between MOM protected

benzyl diethyl phosphonate and different aldehyde groups under a basic condition. The free hydroxyl group was obtained after MOM deprotection using HCl in the second step. After this, the Mannich reaction was performed with paraformaldehyde and Me_2HTACN to form corresponding final “OMe” or “MT” compounds, following the previous reports.[25-26] Interestingly, an additional Mannich reaction on the “MT” compounds can be performed to add another TACN group on the unsubstituted *ortho* position using paraformaldehyde and Me_2HTACN to obtain “DT” compounds. For ZY-15-OMe/MT/DT (Scheme 1C), due to the presence of an aldehyde group on the R2 component of the compound, we modified the synthesis by first forming the double bond with HWE olefination reaction followed by the addition of the benzaldehyde to the compound's backbone using Suzuki coupling reaction. In order to study the effect of double bond's positions on the compounds' properties, we also synthesized ZY-12-OMe/MT/DT (Scheme 1D) with similar structures to ZY-5-OME/MT/DT except for where double bonds are located. Due to the difference in the structures, synthetic steps for ZY-12-OMe/MT/DT were modified accordingly. First, Suzuki coupling was performed between MOM-protected boron pinacol esters and thiophene bromide to form the first intermediate. Then the HWE olefination reaction was performed to synthesize the second intermediate containing the double bond. After that, the MOM deprotection and the Mannich reaction were done to obtain the final "OMe" or "MT" compounds. Similar to the synthesis of other “DT” compounds, ZY-12-DT was synthesized through an additional Mannich reaction. ^{1}H NMR, ^{13}C NMR, HRMS, and detailed synthetic procedures were described in supporting information.



Scheme 1. Synthetic route for the amphiphilic compounds. A) Synthesis for ZY-5-OMe/MT/DT. B) Synthesis for ZY-17-OMe/MT/DT. C) Synthesis for ZY-15-OMe/MT/DT. D) Synthesis for ZY-12-OMe/MT/DT. Reagents and conditions: (1a) KOtBu, DMF, rt, overnight; (1b) HCl, CH_2Cl_2, MeOH, rt, 12 h; (1c) $(CH_2O)n$, Me_2HTACN, MeCN, reflux, 16 h; (1d) $(CH_2O)n$, Me_2HTACN, MeCN, reflux, 24 h; (2a) NaOMe, DMF, rt; (2b) HCl, CH_2Cl_2, MeOH, rt, overnight; (2c) $(CH_2O)_n$, Me_2HTACN, MeCN, reflux; (2d) $(CH_2O)n$, Me_2HTACN, MeCN, reflux, 24 h; (3a) NaOMe, DMF, r, 24 h; (3b) $Pd(PPh_3)_4$, K_2CO_3, toluene, ethanol, reflux; (3c) HCl, CH_2Cl_2, MeOH, rt,12 h; (3d) $(CH_2O)n$, Me_2HTACN, MeCN, reflux, overnight; (3e) $(CH_2O)n$, Me_2HTACN, MeCN, reflux 24 h; (4a) $Pd(PPh_3)_4$, K_2CO_3, toluene, ethanol, reflux, 6 h; (4b) HCl, CH_2Cl_2, MeOH, rt, overnight; (4c) $(CH_2O)n$, Me_2HTACN, MeCN, reflux, 16 h; (4d) $(CH_2O)n$, Me_2HTACN, MeCN, reflux, 24 h.

Fluorescence spectra changes of amphiphilic compounds toward $A\beta_{42}$ fibrils and oligomers

To investigate whether these amphiphilic compounds can interact with different Aβ species, we recorded the fluorescence intensity changes in the absence and presence of two $A\beta_{42}$ aggregates, $A\beta_{42}$ fibrils and oligomers, prepared as previously reported.[27] These compounds, such as ZY-5 series compounds, were observed to have fluorescence turn-on effects when binding to Aβ species, along with a dramatic blue shift of the emission wavelength (Figure 2 and S3). The enhanced fluorescence intensity and blue shift are possibly due to the restriction in a rotation of the aromatic rings and the changes of hydrophobicity at the binding sites.

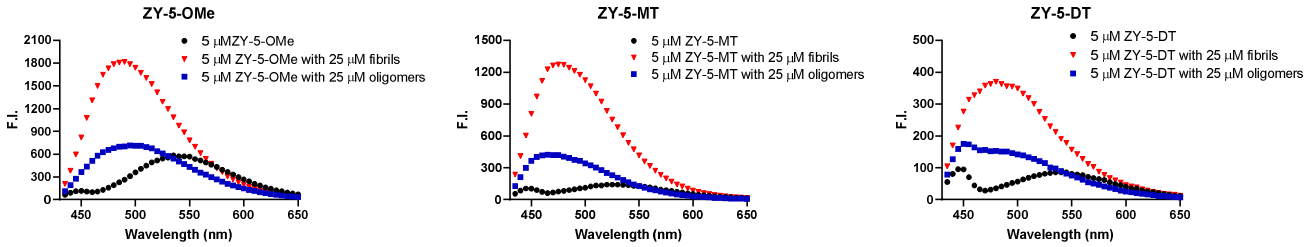


Figure 2. Fluorescence turn-on effects with $A\beta_{42}$ oligomers and fibrils. Left ZY-5-OMe, middle ZY-5-MT right ZY-5-DT. Black: compound only; Blue: compound + $A\beta_{42}$ oligomers; Red: compound + $A\beta_{42}$ fibrils; [compound] = 5 μM; [$A\beta_{42}$ oligomers] = 25 μM; [$A\beta_{42}$ fibrils] = 25 μM.

Binding affinity of amphiphilic compounds toward $A\beta_{42}$ fibrils and oligomers

Inspired by the fluorescence turn-on effect, we measured the binding constants (K_d values) toward $A\beta_{42}$ fibrils and oligomers using fluorescence saturation assays. Representative K_d curves and K_d values obtained for the ZY-5 series are shown in Figure 3 (for other compounds, see Figure S4 and S5). These quantified K_d values (Table 1) for all twelve compounds provide a direct comparison between the compounds' interaction with fibrils and oligomers. It is worth noting that some compounds have excellent binding affinity to oligomers evidenced by the high K_d values (e.g., ZY-17-OMe: K_d = 0.12 μM, ZY-12-OMe: K_d = 0.32 μM. These values are comparable to the K_d values of some reported oligomer-specific probes: higher than CRANAD-102 (7.5 ± 10 nM)[28] but lower than BD-Oligo (K_d = 0.48 μM)[29] and F-SLOH (K_d = 0.66 μM).[30] More interestingly, six of the compounds (ZY-5-MT, ZY-12-OMe, ZY-12-DT, ZY-15-OMe, ZY-17-OMe, and ZY-17-DT) showed

relatively lower K_d values for oligomers than fibrils, indicating their higher binding affinity to oligomers over fibrils. Except for ZY-5-OMe, compounds bearing methoxy group in the other three series all showed higher affinity toward oligomer (ZY-12-OMe, ZY-15-OMe, ZY-17-OMe). This finding demonstrates that the methoxy group might increase the compounds' interactions with Aβ oligomers. Moreover, the ZY-5 series and ZY-12 bind to Aβs differently despite their similar chemical structures. More specifically, ZY-5-MT showed a high affinity to both fibrils and oligomers, with the highest selectivity to oligomers (binding affinity of 3.9 times higher than fibrils). In contrast, ZY-12-MT exhibited low affinity to both fibrils and oligomers. Taken together, we hope the diverse structures along with the detailed K_d data in our study will be beneficial for developing more oligomer-specific compounds in the near future.

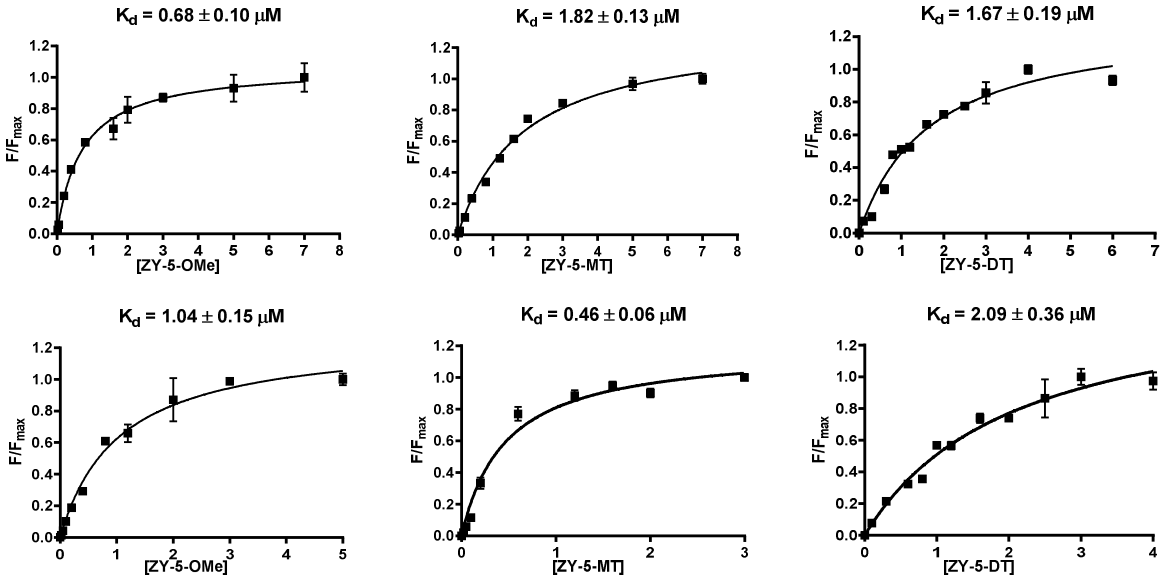


Figure 3. Binding constant measurements of ZY-5-OMe (left), ZY-5-MT (middle), ZY-5-DT (right) with $A\beta_{42}$ fibrils (top), and oligomers (bottom).

Table 1. K_d summary of amphiphilic compounds with $A\beta_{42}$ fibrils and oligomers.

Compounds	Fibrils (μM)	Oligomers (μM)
ZY-5-OMe	0.68 ± 0.10	1.04 ± 0.15
ZY-5-MT	1.82 ± 0.13	0.46 ± 0.06
ZY-5-DT	1.67 ± 0.19	2.09 ± 0.36
ZY-12-OMe	0.39 ± 0.04	0.32 ± 0.04
ZY-12-MT	4.19 ± 0.87	6.27 ± 1.01
ZY-12-DT	1.38 ± 0.24	0.67 ± 0.15
ZY-15-OMe	1.24 ± 0.18	0.47 ± 0.13
ZY-15-MT	0.53 ± 0.03	2.03 ± 0.11
ZY-15-DT	1.27 ± 0.10	4.55 ± 0.32
ZY-17-OMe	0.18 ± 0.02	0.12 ± 0.02
ZY-17-MT	0.94 ± 0.07	2.08 ± 0.34
ZY-17-DT	2.25 ± 0.46	1.03 ± 0.20

Fluorescence Staining of 5xFAD Mouse Brain Sections

In order to confirm that the compounds can also bind to native Aβ species, brain sections collected from 9-month-old 5xFAD transgenic mice have been employed in the following fluorescence staining studies. 5xFAD transgenic mice were shown to develop AD pathologies at a young age, and misfolded Aβ species were commonly observed in brain sections from 5xFAD transgenic mice.[31] The brain sections were first incubated with our compounds and followed by Congo red (CR), which is a well-established fluorescent probe that can bind to Aβ plaques.[32] The treated brain sections were then investigated, and images were taken under a fluorescent microscope. The six compounds with a high binding affinity for oligomers in our previous K_d measurement studies showed well-defined fluorescence staining signals and good colocalization with Congo Red (CR) as indicated by the Pearson's correlation coefficients R-value (Figure 4 and S6). Interestingly, these compounds tend to bind dominantly on the periphery region, while CR binds to the dense core region, which is mainly well-aggregated plaques. Immunostaining with the CF594-conjugated HJ 3.4 antibody (CF594-HJ3.4) was also investigated to confirm that these six compounds can bind to Aβ species specifically on the brain sections (Figure 5). Moreover, the Log D values of the compounds were also measured by octanol-PBS partition assays.[33] These twelve compounds exhibit similar Log D values ranging from 0.8 to 1.2, indicating their potential of passing Blood-Brain Barrier (Table S2).[34-35]

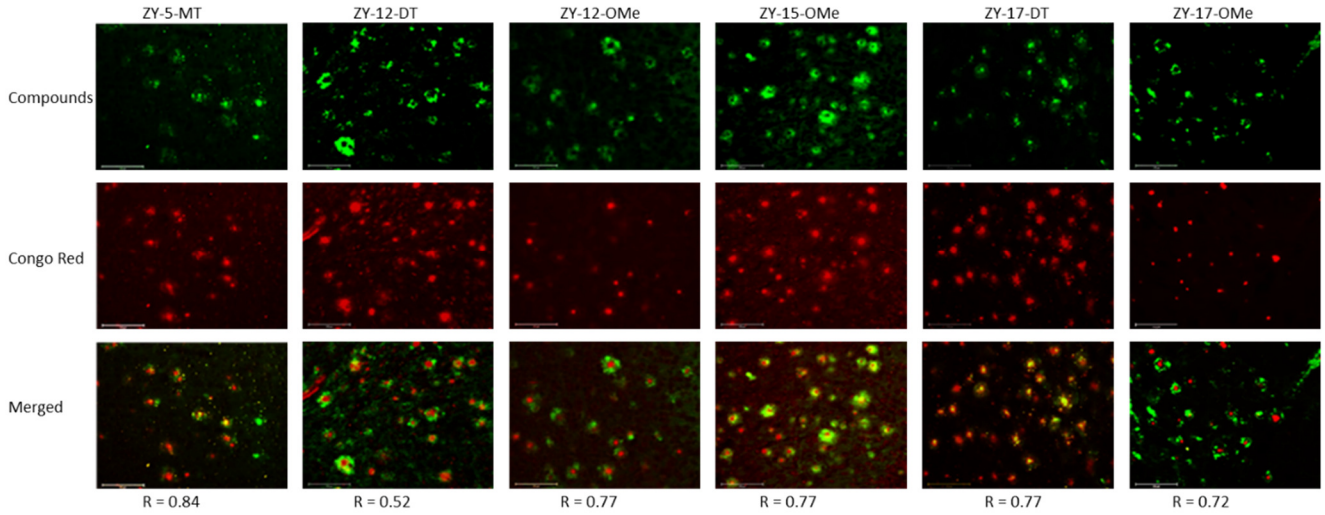


Figure 4. Fluorescence microscopy images of 5xFAD mice brain sections co-incubated with amphiphilic compounds (top), Congo Red (middle) and merged images (bottom, along with the Parson's correlation coefficients R). Concentrations: [amphiphilic compounds] = 5 μM, [Congo Red] = 2.5 μM (scale bar: 125 μm).

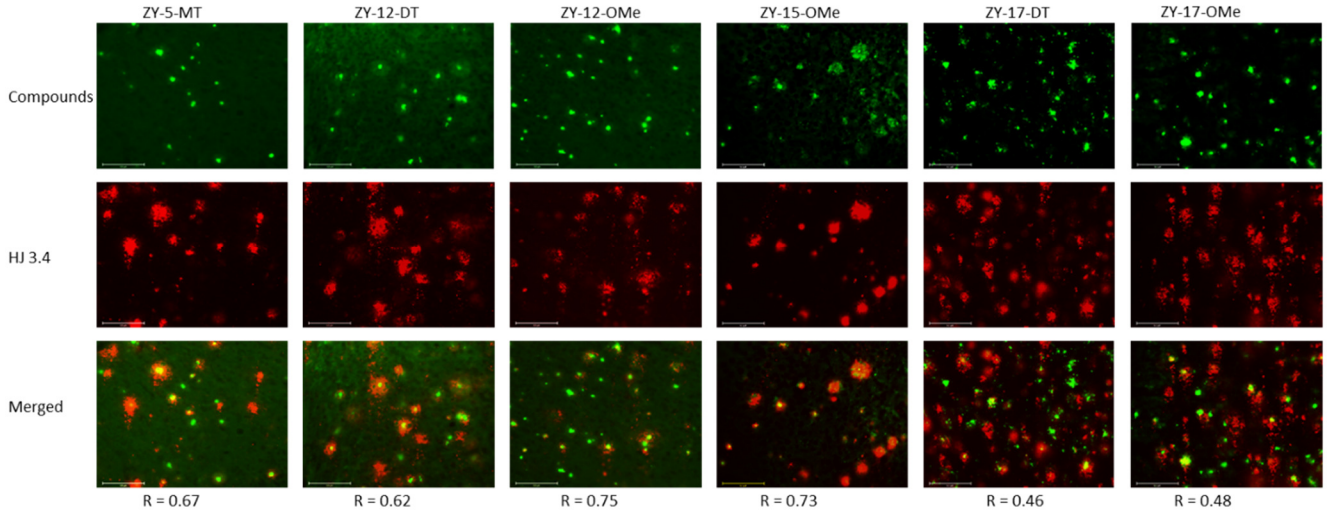


Figure 5. Fluorescence microscopy images of 5xFAD mice brain sections co-incubated with amphiphilic compounds (top), HJ 3.4 (middle) and merged images (bottom, along with the Parson's correlation coefficients R). Concentrations: [amphiphilic compounds] = 5 μM, [CF594-HJ 3.4]: 1 μg/mL (scale bar: 125 μm).

Molecular Docking Studies

To better understand why some compounds showed a higher binding affinity to $A\beta_{42}$ oligomers than fibrils, a series of docking studies using the Schrödinger program Glide were performed.[36] Gunnar F. Schröder and coworkers reported one $A\beta_{42}$ fibrils structure (PDB ID: 5OQV) obtained by cryo-electron microscopy in 2017, and the structure had been widely used as a molecular docking model to study ligands and $A\beta_{42}$ fibrils interactions ever since.[37-39] The protein

structures for $A\beta_{42}$ oligomers are rare due to their heterogeneous and aggregation-prone nature. Recently, $A\beta_{42}$ tetramer (PDB ID: 6RHY) in membrane-mimicking conditions were prepared and reported based on NMR spectroscopy and mass spectrometry by Natàlia Carulla and coworkers.[40] According to their molecular dynamic studies, the tetramer dimerizes and forms Aβ pore-like structures within the membranes. This structure could be a critical model for understanding how the compounds disrupt the interactions between the toxic oligomers and cell membranes. Compounds ZY-5-MT, ZY-12-DT, ZY-15-OMe, ZY-17-DT, and ZY-17-OMe are discussed in this section due to their higher binding affinity toward oligomers than fibrils. ZY-12-OMe is not mentioned since it only showed a slightly higher binding affinity to oligomers. When compounds ZY-5-MT, ZY-15-OMe, and ZY-12-DT were docked onto $A\beta_{42}$ fibrils' structure, neither hydrogen bond nor π-π interaction was observed. Instead, these compounds bind with $A\beta_{42}$ fibrils by inserting into the pocket formed by the residues KLVFF and residues NKGAI (Figure 6a), similar to Thioflavin T – a well-known compound used for Aβ plaques/fibrils binding.[41] Interestingly, ZY-17-DT and ZY-17-OMe can bind to the C-terminal from one Aβ peptide and N-terminal from another Aβ peptide (Figure 6b and c). More specifically, they can form hydrogen bonds and salt bridges with the amino acids Asp1 and Ala42. Their binding motif is similar to that of the fluorescent probe reported by the Ran group, which can differentiate $A\beta_{42}$ fibrils and $A\beta_{40}$ fibrils by targeting the extra two amino acids at the C terminal of the $A\beta_{42}$ fibrils, suggesting that ZY-17-DT and ZY-17-OMe might also have a different binding affinity towards $A\beta_{42}$ vs. $A\beta_{40}$ fibrils.[42]

When considering the docking results with $A\beta_{42}$ tetramers, the compounds exhibit more interactions with the oligomers' structure. ZY-5-MT, ZY-17-OMe, and ZY-17-DT all interact with His6 and Asp7 via hydrogen bonds and salt bridges (Figure 7a, c, and d). On the other hand, ZY-12-DT interacts with Phe4 and Asp7 instead of His6 and Asp7 (Figure 7b). ZY-15-OMe binds to another amino acid – Tyr10 – through hydrogen bonds and π-π interactions (Figure 7e). Since both compound ZY-5-MT (with the most selectivity toward oligomers) and compound ZY-17-OMe

(which has the highest binding affinity to oligomers) showed interactions with amino acids His6 and Asp7, we believe that targeting these two residues would increase the binding affinity and selectivity to Aβ oligomers.

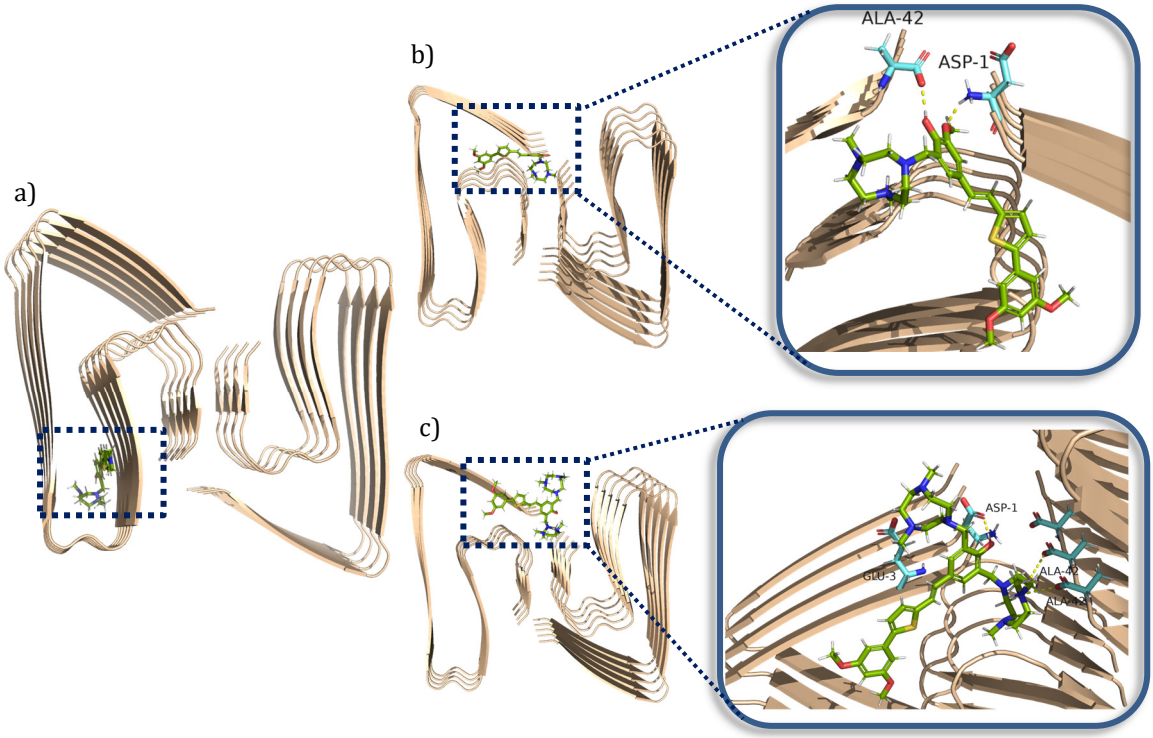


Figure 6. Calculated binding modes of amphiphilic compounds to $A\beta_{42}$ fibrils structure (5OQV). a) Representative pose of ZY-5-MT, ZY-15-OMe, and ZY-12-DT. b) ZY-17-OMe. c) ZY-17-DT.

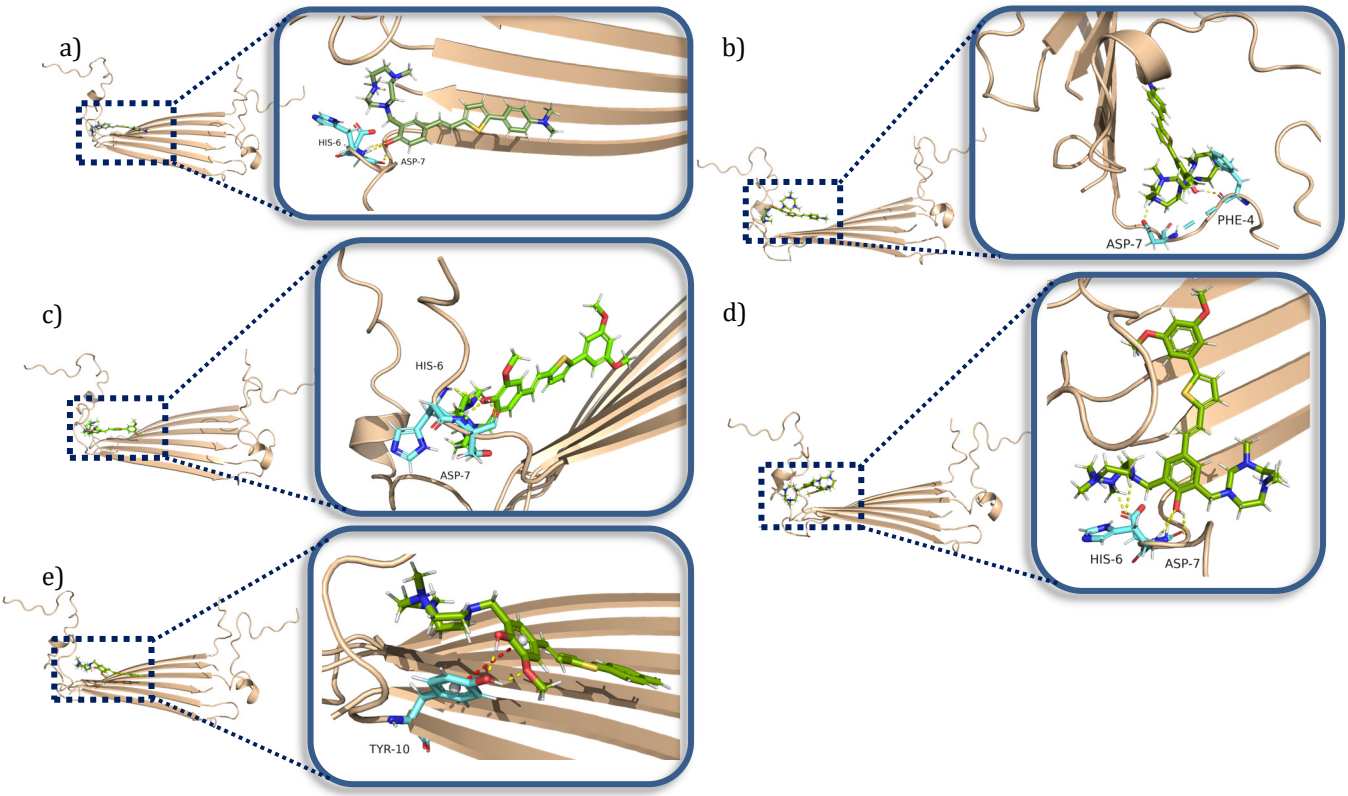


Figure 7. Calculated binding modes of amphiphilic compounds to $A\beta_{42}$ tetramer structure (6RHY). a) ZY-5-MT b) ZY-12-DT c) ZY-17-OMe d) ZY-17-DT f) ZY-15-OMe.

Cytotoxicity of the amphiphilic compounds and modulation of Cu^{2+}-$A\beta_{42}$ neurotoxicity

Confirming these amphiphilic compounds can bind to Aβs both *in vitro* and *ex vivo*, we next investigated whether the compounds could attenuate the toxicity induced by the Cu-Aβ complex as the Cu^{2+} ions were reported to promote the formation of neurotoxic soluble $A\beta_{42}$ oligomers.[43-44] Firstly, the compounds' neurotoxicity was evaluated. The Alamar blue cell viability assay was used to measure the compounds' cytotoxicity at different concentrations ranging from 20 μM to 2 μM in mouse neuroblastoma N2a cells (Figure 8). Some compounds (ZY-12-MT, ZY-15-MT, ZY-15-OMe, ZY-17-MT, ZY-5-MT, ZY-5-DT, and ZY-5-OMe) exhibited no significant cytotoxicity (indicated by >80% cell viability) up to 10 μM. Hence, these compounds are good candidates for the Cu^{2+}-$A\beta_{42}$-induced cytotoxicity studies (see below). For ZY-12-OMe, the compound was quite toxic even at 10 μM (with cell viability less than 50%), yet at 5 μM it exhibited less pronounced cytotoxicity (more than 75% cell viability). Considering its high binding affinity to both Aβ fibrils and oligomers, we

also further tested its ability to alleviate Cu^{2+}-$A\beta_{42}$-induced toxicity. Unfortunately, some compounds showed a high binding affinity to oligomers, such as ZY-12-OMe, ZY-12-DT, ZY-17-DT, and ZY-17-OMe, exhibited higher toxicity than others. Additionally, ZY-17-OMe, which has the highest binding affinity to oligomers, showed the highest toxicity. The underlying mechanism is unclear, but we propose that these compounds might perform similarly to the toxic oligomers that bind to some cell membrane receptors or insert into the membrane lipids to form porous channels.

Since our compounds exhibit high binding affinity to both Aβ fibrils/oligomers and contain TACN group(s) that can potentially bind to Cu and disrupt the Cu–$A\beta_{42}$ interaction, [45] it is essential to study our compounds' roles in alleviating Cu^{2+}-$A\beta_{42}$-induced toxicity. As we mentioned above, compounds that didn't exhibit apparent cytotoxicity at 10 μM, including ZY-12-MT, ZY-15-MT, ZY-15-OMe, ZY-17-MT, ZY-5-MT, ZY-5-DT, ZY-5-OMe, and ZY-12-OMe, were chosen for this study. Firstly, during the control studies, we observed monomeric $A\beta_{42}$ led to negligible neurotoxicity. Nevertheless, in the presence of both Cu^{2+} and monomeric $A\beta_{42}$, there was a significant cell death, which is probably due to the Cu^{2+} associated neurotoxic $A\beta_{42}$ oligomers formation. We observed compounds ZY-12-MT, ZY-15-OMe, ZY-15-MT, and ZY-5-OMe could significantly increase cell viability while the other ones could not reduce the neurotoxicity of the Cu^{2+}-$A\beta_{42}$ species (Figure 9). Interestingly, compared to ZY-5-MT, even though ZY-15-OMe is less selective toward oligomers, it can alleviate Cu^{2+}-$A\beta_{42}$-induced toxicity probably due to its interactions with Tyr10 via hydrogen bond and π-π interaction. According to the docking results, ZY-12-MT, ZY-15-OMe, ZY-15-MT, and ZY-5-OMe interact with $A\beta_{42}$ tetramers mainly through residues His6, Asp7, Tyr10 (via hydrogen bonds), and Tyr10 (via π-π interaction). Some reports have also shown that His6, Asp7, and Tyr10 are potentially involved in the Cu-Aβ interactions, which explained why the compounds are able to attenuate the Cu-Aβ toxicity. [46]

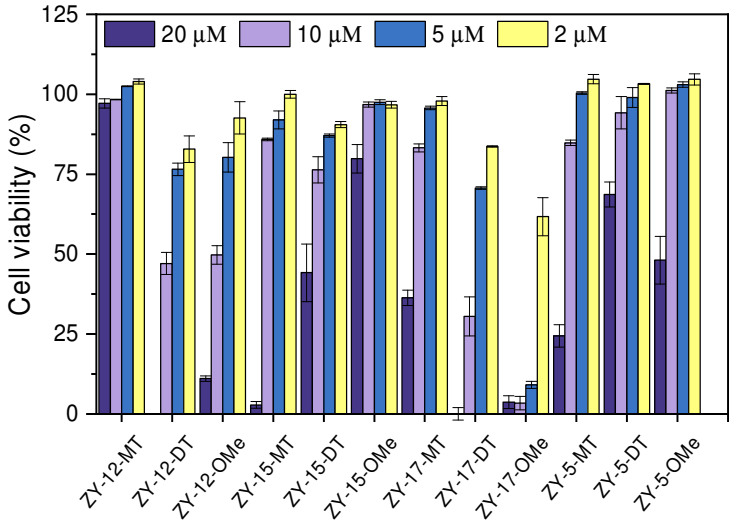


Figure 8. Toxicity of the amphiphilic compounds with different concentrations in mouse neuroblastoma Neuro2A (N2a) cells.

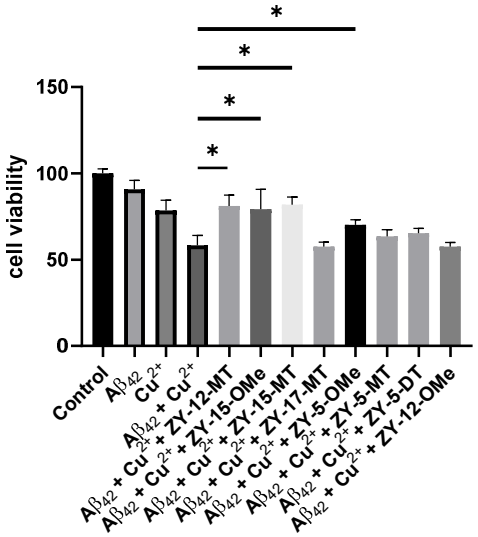


Figure 9. Cell viability results upon incubation of Neuro2A cells with monomeric $A\beta_{42}$ in the presence or absence of metal ions and amphiphilic compounds. Concentrations: [$A\beta_{42}$] = 40 μM, [Cu^{2+}] = 40 μM, [ZY-12-MT] = [ZY-15-OMe] = [ZY-15-MT] = [ZY-17-MT] = [ZY-5-OMe] = [ZY-5-MT] = [ZY-5-DT] = 10 μM, [ZY-12-OMe] = 5 μM. The error bars represent the standard deviation from five independent experiments, and the statistical analysis was evaluated according to one-way ANOVA (*$p < 0.05$).

Controlling the Aβ-cell membranes interactions with the amphiphilic compounds

Encouraged by the compounds' ability to attenuate the neurotoxicity of Cu-Aβ species, we sought out to study the possible molecular mechanisms. Aβ oligomers were reported to interact with cell membranes in various ways, such as binding to receptors on cell membranes,[47-48] inserting into membranes,[49] or even showing cellular uptake via endocytosis process.[50] The abnormal interactions between Aβ oligomers and neuron cells, which could disrupt the neuronal ion

homeostasis and neuron cell membrane integrity, might be why Aβ oligomers are highly neurotoxic.[51-53] Moreover, molecules that can disrupt interactions between oligomers and cells are promising candidate for drug development.[54-55] Taking advantage of the confocal imaging technique, we were able to probe the interaction of the $A\beta_{42}$ oligomers with SH-SY5Y cellular membranes in the absence and presence of the compounds. Because ZY-15-MT and ZY-15-OMe can recure the cells to a higher extent and exhibit a higher affinity to oligomers than ZY-12-MT, they were chosen for this experiment. SH-SY5Y cells were treated with oligomers or a combination of oligomers and compounds for 24 h, followed by the immunofluorescence staining with anti-Aβ antibody HJ 3.4 (labeled directly with CF594) and nuclei staining, shown in the red and blue channel, respectively. Compared to the untreated group, oligomers were found mainly bound to cell membranes. Moreover, in the presence of both ZY-15-MT and ZY-15-OMe, there were fewer numbers of oligomers bound to the cell membranes, as shown in the red channel (Figure 10a). Interesting, ZY-15-MT is able to decrease the numbers of the oligomers binding to the membranes to a larger extent (about 50%, Figure 10b), even though ZY-15-MT shows a lower affinity to oligomers than ZY-15-OMe. We believe that the oligomers also start the fibrilization process when incubated with cells. ZY-15-MT, which exhibits a higher affinity to fibrils than ZY-15-OMe, might be able to bind to the more aggregated Aβ species and can also decrease their interactions with cell membranes. These findings suggest that the amphiphilic compounds are neuroprotective by controlling the interactions between oligomers and cell membranes.

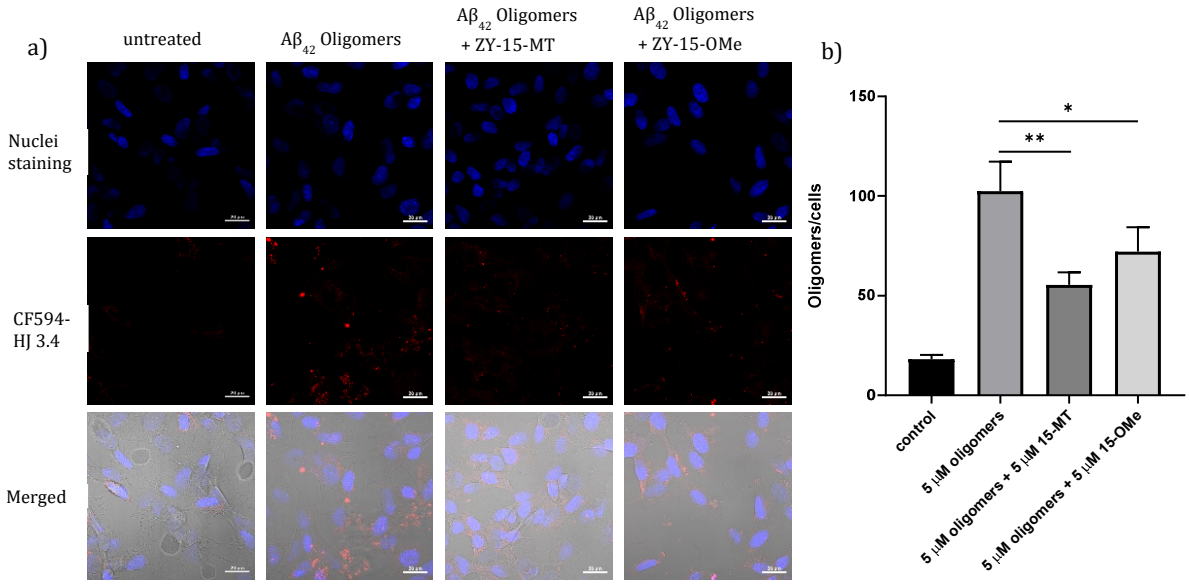


Figure 10. a) SH-SY5Y cells were treated with 5 μM oligomers in the presence or absence of 5 μM ZY-15-MT or 5 μM ZY-15-OMe for 24 h hours before imaging. Red and blue fluorescence indicate the oligomers and nuclei, respectively. Scar bar, 20 μm. b) In each experiment, 7 to 10 images were taken, and a total of 150-200 cells per experiment were analyzed. Three independent experiments were subjected for the statistical analysis and analyzed by one-way ANOVA (*P < 0.05, **P < 0.01).

Conclusions

In conclusion, we designed and synthesized 12 amphiphilic compounds and studied their binding affinity toward $A\beta_{42}$ species *in vitro* and *ex vivo* in the AD mice brain sections. More importantly, according to the molecular docking studies, we found targeting amino acids His6 and Asp7 might increase the binding affinity and selectivity toward $A\beta_{42}$ oligomers. Moreover, according to our cellular studies, we found that compounds with a higher binding affinity toward $A\beta_{42}$ oligomers exhibited higher neurotoxicity, and most of them interact mainly with amino acids His6 and Asp7. The only exception is compound ZY-15-OMe, which maintains its selectivity to oligomers via hydrogen bonds and π-π interaction with Tyr10, shows less neurotoxicity by itself and alleviates Cu^{2+}-$A\beta_{42}$-induced toxicity. Finally, confocal microscope studies proved both ZY-15-MT and ZY-15-OMe were able to decrease the interactions between oligomers and SH-SY5Y cell membranes, demonstrating their abilities to target Aβ oligomers. These studies showed that developing amphiphilic compounds could be an effective strategy to differentiate Aβ oligomers and

fibrils. We believe these encouraging results will help design less neurotoxic, oligomers-specific therapeutic reagents, which can be used for further application in AD treatment.

Conflicts of interest

The authors declare no competing financial interest.

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References

1. Hardy, J.; Selkoe, D. J., The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **2002,** *297* (5580), 353-6.
2. Selkoe, D. J.; Hardy, J., The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med* **2016,** *8* (6), 595-608.
3. Spillantini, M. G.; Schmidt, M. L.; Lee, V. M. Y.; Trojanowski, J. Q.; Jakes, R.; Goedert, M., α-Synuclein in Lewy bodies. *Nature* **1997,** *388* (6645), 839-840.
4. 2020 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **2020,** *16* (3), 391-460.
5. Selkoe, D. J., Resolving controversies on the path to Alzheimer's therapeutics. *Nat. Med.* **2011,** *17* (9), 1060-1065.
6. Haass, C.; Selkoe, D. J., Soluble Protein Oligomers in Neurodegeneration: Lessons from the Alzheimer's Amyloid β-Peptide. *Nat. Rev. Mol. Cell Biol.* **2007,** *8* (2), 101–112.
7. Benilova, I.; Karran, E.; De Strooper, B., The Toxic Aβ Oligomer and Alzheimer's Disease: An Emperor in Need of Clothes. *Nat. Neurosci.* **2012,** *15*, 349-357.
8. Lee, S. J. C.; Nam, E.; Lee, H. J.; Savelieff, M. G.; Lim, M. H., Towards an understanding of amyloid-beta oligomers: characterization, toxicity mechanisms, and inhibitors. *Chem. Soc. Rev.* **2017,** *46* (2), 310-323.

9. Cline, E. N.; Bicca, M. A.; Viola, K. L.; Klein, W. L., The Amyloid-β Oligomer Hypothesis: Beginning of the Third Decade. *J. Alzheimer's Dis.* **2018,** *64*, S567-S610.
10. Karran, E.; Mercken, M.; Strooper, B. D., The Amyloid Cascade Hypothesis for Alzheimer's Disease: An Appraisal for the Development of Therapeutics. *Nat. Rev. Drug Discovery* **2011,** *10* (9), 698-712.
11. Hefti, F.; Goure, W. F.; Jerecic, J.; Iverson, K. S.; Walicke, P. A.; Krafft, G. A., The case for soluble Aβ oligomers as a drug target in Alzheimer's disease. *Trends Pharmacol. Sci.* **2013,** *34* (5), 261-266.
12. Ladiwala, A. R. A.; Bhattacharya, M.; Perchiacca, J. M.; Cao, P.; Raleigh, D. P.; Abedini, A.; Schmidt, A. M.; Varkey, J.; Langen, R.; Tessier, P. M., Rational design of potent domain antibody inhibitors of amyloid fibril assembly. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** *109* (49), 19965.
13. Xiao, C.; Davis, F. J.; Chauhan, B. C.; Viola, K. L.; Lacor, P. N.; Velasco, P. T.; Klein, W. L.; Chauhan, N. B., Brain transit and ameliorative effects of intranasally delivered anti-amyloid-β oligomer antibody in 5XFAD mice. *J. Alzheimer's Dis.* **2013,** *35* (4), 777-788.
14. Frydman-Marom, A.; Rechter, M.; Shefler, I.; Bram, Y.; Shalev, D. E.; Gazit, E., Cognitive-Performance Recovery of Alzheimer's Disease Model Mice by Modulation of Early Soluble Amyloidal Assemblies. *Angew. Chem., Int. Ed.* **2009,** *48* (11), 1981-1986.
15. Salveson, P. J.; Haerianardakani, S.; Thuy-Boun, A.; Kreutzer, A. G.; Nowick, J. S., Controlling the Oligomerization State of Aβ-Derived Peptides with Light. *J. Am. Chem. Soc.* **2018,** *140* (17), 5842-5852.
16. Ehrnhoefer, D. E.; Bieschke, J.; Boeddrich, A.; Herbst, M.; Masino, L.; Lurz, R.; Engemann, S.; Pastore, A.; Wanker, E. E., EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat. Struct. Mol. Biol.* **2008,** *15* (6), 558-566.
17. Ladiwala, A. R. A.; Dordick, J. S.; Tessier, P. M., Aromatic small molecules remodel toxic soluble oligomers of amyloid beta through three independent pathways. *J. Biol. Chem* **2011,** *286* (5), 3209-3218.
18. Lenhart, J. A.; Ling, X.; Gandhi, R.; Guo, T. L.; Gerk, P. M.; Brunzell, D. H.; Zhang, S., "Clicked" Bivalent Ligands Containing Curcumin and Cholesterol As Multifunctional Aβ Oligomerization Inhibitors: Design, Synthesis, and Biological Characterization. *J. Med. Chem.* **2010,** *53* (16), 6198-6209.
19. Sun, L.; Sharma, A. K.; Han, B.-H.; Mirica, L. M., Amentoflavone: A Bifunctional Metal Chelator that Controls the Formation of Neurotoxic Soluble Aβ42 Oligomers. *ACS Chem. Neurosci.* **2020,** *11* (17), 2741-2752.
20. Bieschke, J.; Herbst, M.; Wiglenda, T.; Friedrich, R. P.; Boeddrich, A.; Schiele, F.; Kleckers, D.; Lopez del Amo, J. M.; Grüning, B. A.; Wang, Q.; Schmidt, M. R.; Lurz, R.; Anwyl, R.; Schnoegl, S.; Fändrich, M.; Frank, R. F.; Reif, B.; Günther, S.; Walsh, D. M.; Wanker, E. E., Small-molecule conversion of toxic oligomers to nontoxic β-sheet–rich amyloid fibrils. *Nat. Chem. Biol.* **2012,** *8* (1), 93-101.
21. Freskgård, P.-O.; Urich, E., Antibody therapies in CNS diseases. *Neuropharm.* **2017,** *120*, 38-55.

22. Maity, D.; Howarth, M.; Vogel, M. C.; Magzoub, M.; Hamilton, A. D., Peptidomimetic-Based Vesicles Inhibit Amyloid-β Fibrillation and Attenuate Cytotoxicity. *J. Am. Chem. Soc.* **2021,** *143* (8), 3086-3093.
23. Sun, L.; Cho, H.-J.; Sen, S.; Arango, A. S.; Bandara, N.; Huang, Y.; Huynh, T. T.; Rogers, B. E.; Tajkhorshid, E.; Mirica, L. M., Amphiphilic Distyrylbenzene Derivatives as Potential Therapeutic and Imaging Agents for the Soluble Amyloid-β Oligomers in Alzheimer's Disease. *ChemRxiv preprint: DOI: 10.26434/chemrxiv.13605998*.
24. Necula, M.; Kayed, R.; Milton, S.; Glabe, C. G., Small molecule inhibitors of aggregation indicate that amyloid β oligomerization and fibrillization pathways are independent and distinct. *J. Biol. Chem.* **2007,** *282* (14), 10311–10324.
25. Bandara, N.; Sharma, A. K.; Krieger, S.; Schultz, J. W.; Han, B. H.; Rogers, B. E.; Mirica, L. M., Evaluation of ^{64}Cu-based Radiopharmaceuticals That Target Aβ Peptide Aggregates as Diagnostic Tools for Alzheimer's Disease. *J. Am. Chem. Soc.* **2017,** *139* (36), 12550-12558.
26. Sharma, A. K.; Schultz, J. W.; Prior, J. T.; Rath, N. P.; Mirica, L. M., Coordination Chemistry of Bifunctional Chemical Agents Designed for Applications in (64)Cu PET Imaging for Alzheimer's Disease. *Inorg. Chem.* **2017,** *56* (22), 13801-13814.
27. Klein, W. L.; Krafft, G. A.; Finch, C. E., Targeting small A beta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci.* **2001,** *24* (4), 219-224.
28. Li, Y.; Yang, J.; Liu, H.; Yang, J.; Du, L.; Feng, H.; Tian, Y.; Cao, J.; Ran, C., Tuning the stereo-hindrance of a curcumin scaffold for the selective imaging of the soluble forms of amyloid beta species. *Chem. Sci.* **2017,** *8* (11), 7710-7717.
29. Teoh, C. L.; Su, D.; Sahu, S.; Yun, S. W.; Drummond, E.; Prelli, F.; Lim, S.; Cho, S.; Ham, S.; Wisniewski, T.; Chang, Y. T., Chemical Fluorescent Probe for Detection of Abeta Oligomers. *J. Am. Chem. Soc.* **2015,** *137* (42), 13503-9.
30. Li, Y.; Xu, D.; Sun, A.; Ho, S. L.; Poon, C. Y.; Chan, H. N.; Ng, O. T. W.; Yung, K. K. L.; Yan, H.; Li, H. W.; Wong, M. S., Fluoro-substituted cyanine for reliable in vivo labelling of amyloid-beta oligomers and neuroprotection against amyloid-beta induced toxicity. *Chem. Sci.* **2017,** *8* (12), 8279-8284.
31. Oakley, H.; Cole, S. L.; Logan, S.; Maus, E.; Shao, P.; Craft, J.; Guillozet-Bongaarts, A.; Ohno, M.; Disterhoft, J.; Van Eldik, L.; Berry, R.; Vassar, R., Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci* **2006,** *26* (40), 10129-40.
32. Wilcock, D. M.; Gordon, M. N.; Morgan, D., Quantification of cerebral amyloid angiopathy and parenchymal amyloid plaques with Congo red histochemical stain. *Nat. Protoc.* **2006,** *1*, 1591–1595.
33. Ran, C. Z.; Xu, X. Y.; Raymond, S. B.; Ferrara, B. J.; Neal, K.; Bacskai, B. J.; Medarova, Z.; Moore, A., Design, Synthesis, and Testing of Difluoroboron-Derivatized Curcumins as Near-Infrared Probes for in Vivo Detection of Amyloid-beta Deposits. *J. Am. Chem. Soc.* **2009,** *131* (42), 15257-15261.
34. Wager, T. T.; Chandrasekaran, R. Y.; Hou, X.; Troutman, M. D.; Verhoest, P. R.; Villalobos, A.; Will, Y., Defining Desirable Central Nervous System Drug Space through the Alignment of

Molecular Properties, in Vitro ADME, and Safety Attributes. *ACS Chem. Neurosci.* **2010,** *1* (6), 420-434.
35. Wager, T. T.; Hou, X.; Verhoest, P. R.; Villalobos, A., Moving beyond Rules: The Development of a Central Nervous System Multiparameter Optimization (CNS MPO) Approach to Enable Alignment of Druglike Properties. *ACS Chem. Neurosci.* **2010,** *1* (6), 435-449.
36. Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S., Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J. Med. Chem.* **2004,** *47* (7), 1739-49.
37. Gremer, L.; Scholzel, D.; Schenk, C.; Reinartz, E.; Labahn, J.; Ravelli, R. B. G.; Tusche, M.; Lopez-Iglesias, C.; Hoyer, W.; Heise, H.; Willbold, D.; Schroder, G. F., Fibril structure of amyloid-beta(1-42) by cryo-electron microscopy. *Science* **2017,** *358* (6359), 116-119.
38. Zou, Y.; Qian, Z.; Chen, Y.; Qian, H.; Wei, G.; Zhang, Q., Norepinephrine Inhibits Alzheimer's Amyloid-β Peptide Aggregation and Destabilizes Amyloid-β Protofibrils: A Molecular Dynamics Simulation Study. *ACS Chem. Neurosci.* **2019,** *10* (3), 1585-1594.
39. Gautieri, A.; Beeg, M.; Gobbi, M.; Rigoldi, F.; Colombo, L.; Salmona, M., The Anti-Amyloidogenic Action of Doxycycline: A Molecular Dynamics Study on the Interaction with Aβ42. *Int J Mol Sci* **2019,** *20* (18).
40. Ciudad, S.; Puig, E.; Botzanowski, T.; Meigooni, M.; Arango, A. S.; Do, J.; Mayzel, M.; Bayoumi, M.; Chaignepain, S.; Maglia, G.; Cianferani, S.; Orekhov, V.; Tajkhorshid, E.; Bardiaux, B.; Carulla, N., A beta(1-42) tetramer and octamer structures reveal edge conductivity pores as a mechanism for membrane damage. *Nat. Commun.* **2020,** *11* (1).
41. Biancalana, M.; Koide, S., Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim. Biophys. Acta* **2010,** *1804* (7), 1405-1412.
42. Yang, J.; Zhu, B.; Yin, W.; Han, Z.; Zheng, C.; Wang, P.; Ran, C., Differentiating Aβ40 and Aβ42 in amyloid plaques with a small molecule fluorescence probe. *Chem. Sci.* **2020,** *11* (20), 5238-5245.
43. Hindo, S. S.; Mancino, A. M.; Braymer, J. J.; Liu, Y. H.; Vivekanandan, S.; Ramamoorthy, A.; Lim, M. H., Small Molecule Modulators of Copper-Induced A beta Aggregation. *J. Am. Chem. Soc.* **2009,** *131* (46), 16663-16664.
44. Sharma, A. K.; Pavlova, S. T.; Kim, J.; Kim, J.; Mirica, L. M., The effect of Cu^{2+} and Zn^{2+} on the $A\beta_{42}$ peptide aggregation and cellular toxicity. *Metallomics* **2013,** *5* (11), 1529-1536.
45. Huang, Y.; Cho, H.-J.; Bandara, N.; Sun, L.; Tran, D.; Rogers, B. E.; Mirica, L. M., Metal-chelating benzothiazole multifunctional compounds for the modulation and 64Cu PET imaging of Aβ aggregation. *Chem. Sci.* **2020,** *11* (30), 7789-7799.
46. Atrian-Blasco, E.; Gonzalez, P.; Santoro, A.; Alies, B.; Faller, P.; Hureau, C., Cu and Zn coordination to amyloid peptides: From fascinating chemistry to debated pathological relevance. *Coord. Chem. Rev.* **2018,** *375*, 38-55.
47. Talantova, M.; Sanz-Blasco, S.; Zhang, X.; Xia, P.; Akhtar, M. W.; Okamoto, S.-i.; Dziewczapolski, G.; Nakamura, T.; Cao, G.; Pratt, A. E.; Kang, Y.-J.; Tu, S.; Molokanova, E.; McKercher, S. R.; Hires, S. A.; Sason, H.; Stouffer, D. G.; Buczynski, M. W.; Solomon, J. P.; Michael, S.; Powers, E. T.; Kelly, J. W.; Roberts, A.; Tong, G.; Fang-Newmeyer, T.; Parker, J.;

Holland, E. A.; Zhang, D.; Nakanishi, N.; Chen, H. S. V.; Wolosker, H.; Wang, Y.; Parsons, L. H.; Ambasudhan, R.; Masliah, E.; Heinemann, S. F.; Piña-Crespo, J. C.; Lipton, S. A., Aβ induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss. *Proc. Natl. Acad. Sci. U. S. A.* **2013,** *110* (27), E2518.

48. Um, J. W.; Nygaard, H. B.; Heiss, J. K.; Kostylev, M. A.; Stagi, M.; Vortmeyer, A.; Wisniewski, T.; Gunther, E. C.; Strittmatter, S. M., Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat. Neurosci.* **2012,** *15* (9), 1227-U85.
49. Cerf, E.; Sarroukh, R.; Tamamizu-Kato, S.; Breydo, L.; Derclaye, S.; Dufrêne, Yves F.; Narayanaswami, V.; Goormaghtigh, E.; Ruysschaert, J.-M.; Raussens, V., Antiparallel β-sheet: a signature structure of the oligomeric amyloid β-peptide. *Biochem. J.* **2009,** *421* (3), 415-423.
50. Lai, A. Y.; McLaurin, J., Mechanisms of amyloid-Beta Peptide uptake by neurons: the role of lipid rafts and lipid raft-associated proteins. *Int J Alzheimers Dis* **2010,** *2011*, 548380-548380.
51. Arispe, N.; Rojas, E.; Pollard, H. B., Alzheimer-Disease Amyloid Beta-Protein Forms Calcium Channels in Bilayer-Membranes - Blockade by Tromethamine and Aluminum. *Proc. Natl. Acad. Sci. U. S. A.* **1993,** *90* (2), 567-571.
52. Arispe, N.; Pollard, H. B.; Rojas, E., Zn2+ interaction with Alzheimer amyloid beta protein calcium channels. *Proc. Natl. Acad. Sci. U. S. A.* **1996,** *93* (4), 1710-1715.
53. Lin, H.; Bhatia, R.; Lal, R., Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology. *FASEB J.* **2001,** *15* (13), 2433-2444.
54. Limbocker, R.; Chia, S.; Ruggeri, F. S.; Perni, M.; Cascella, R.; Heller, G. T.; Meisl, G.; Mannini, B.; Habchi, J.; Michaels, T. C. T.; Challa, P. K.; Ahn, M.; Casford, S. T.; Fernando, N.; Xu, C. K.; Kloss, N. D.; Cohen, S. I. A.; Kumita, J. R.; Cecchi, C.; Zasloff, M.; Linse, S.; Knowles, T. P. J.; Chiti, F.; Vendruscolo, M.; Dobson, C. M., Trodusquemine enhances Aβ42 aggregation but suppresses its toxicity by displacing oligomers from cell membranes. *Nat. Commun.* **2019,** *10* (1), 225.
55. Grayson, J. D.; Baumgartner, M. P.; Santos Souza, C. D.; Dawes, S. J.; El Idrissi, I. G.; Louth, J. C.; Stimpson, S.; Mead, E.; Dunbar, C.; Wolak, J.; Sharman, G.; Evans, D.; Zhuravleva, A.; Roldan, M. S.; Colabufo, N. A.; Ning, K.; Garwood, C.; Thomas, J. A.; Partridge, B. M.; de la Vega de Leon, A.; Gillet, V. J.; Rauter, A. P.; Chen, B., Amyloid binding and beyond: a new approach for Alzheimer's disease drug discovery targeting Aβo–PrPC binding and downstream pathways. *Chem. Sci.* **2021,** *12* (10), 3768-3785.