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In Vitro Studies on Accelerating the Degradation and Clearance of Amyloid-β Fibrils by an Antiamyloidogenic Peptide

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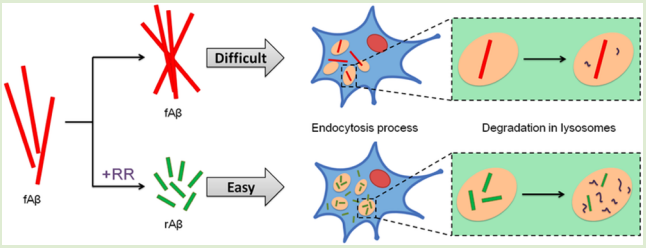
Supporting Information

ABSTRACT: The clearance of overloaded amyloid-β (Aβ) species, especially the toxic aggregates, was thought to be an attractive and promising strategy for Alzheimer's disease (AD) therapy in the past decade. In this work, an active Aβ inhibitor decapeptide RR was used to transform mature Aβ fibrils (fAβ) into nanorod-like Aβ assemblies (rAβ) as well as loosen the β-structure of rAβ. Compared with fAβ, rAβ could be engulfed by PC12 cells more efficiently and showed a 1.46-fold difference. More importantly, the rAβ was colocated with lysosomes after endocytosis, and in vitro study illustrated that rAβ were easily degraded by lysosome protease cathepsin B when compared with the fibrils. Thus, our study indicated the potential application of RR in Aβ fibrils clearance by a cell-participated and enzyme-mediated pathway.



Alzheimer's disease (AD), as a neurodegenerative disorder, is the most common form of dementia in humans. It is widely accepted that the process of amyloid-β peptides (Aβ) self-assembly into soluble oligomers, protofibrils, and finally insoluble mature fibrils with β-sheet structure is the significant step inducing AD. Since the soluble oligomer is the most toxic species,[1−4] many studies have focused on designing various inhibitors, such as small molecules binding with hydrophobic core of Aβ[5] or chelating agents targeting metal-Aβ species,[6−8] against Aβ aggregation and reduce its cytotoxicity successively. However, recent research also suggests that the insufficient clearance rather than the excessive production of Aβ contributes more to its accumulation.[9,10] Therefore, the clearance of overloaded Aβ, especially those with the β-sheet structure, becomes a potential therapeutic strategy for AD.

Cellular engulfment is an important mechanism for clearing the toxic protein in the brain, and once the toxic protein is internalized, it can be degraded by various proteases. Thus, during the past decade, cell-participated and enzyme-mediated degradation of Aβ has attracted a huge amount of attention.[11−13] Xiao et al. found that the transcription factor EB could stimulate the internalization and in turn the degradation of Aβ via lysosomes.[13] It has been also reported that Aβ fibrils (fAβ), via the process of receptor-mediated cell uptake, could be transported within the endosomal-lysosomal pathway and then degraded by proteases such as cathepsin B and D in lysosomes.[14,15] However, due to its big size and intermolecular parallel β-sheets structure, fAβ exhibits relatively lower cellular uptake efficiency and degradation rate than other types of Aβ.[16−18] Consequently, decreasing the size and β-sheets content in aggregates would benefited for increasing the uptake of fAβ and promoting its degradation in lysosome.

In our previous work, an active decapeptide inhibitor RR (RYYAAFFARR) was designed, which enables 75% inhibition of $Aβ_{40}$ fibrillation at an equimolar concentration and a complete inhibition at a molar ratio of 1:4 (Aβ/RR).[19] Herein, we found that RR could also transform the Aβ mature fibril into nanorod-like fragments (rAβ), which is also a less toxic form of Aβ according to our following study. To our knowledge, the rod-like nanoparticles could be easily internalized by the endocytosis and show a more rapid cell uptake rate.[20,21] Therefore, we suppose that compared with fAβ, rAβ could be easier endocytosed by cells into lysosome and in turn degraded by lysosomal enzymes.

To investigate the transformation effect of RR on fAβ, thioflavin-T (ThT) fluorescence assay was first used. As shown in Figure 1a, the fluorescence intensity kept increasing with the incubation of Aβ monomer solution in the first day, then it remained stable in the next 2 days, illustrating that the fibrils were generated. This result was supported by TEM (Figure 1b), consistent with our previous work.[19] After RR added in the fibrils solution, the fluorescence intensity decreased quickly in the first day, indicating the destruction of β-sheet structure. When the curve became stable after 2 days coincubation, the ThT fluorescence intensity of Aβ fibrils decreased about 45% both with a molar ratio of 1:1 and 1:5 (Aβ/RR). Since the reduction of fluorescence intensity may result from the competitive binding between RR and ThT toward the fibrils, circular dichroism (CD) was used for confirming it. Fortunately, the decrease of β-sheet structure in fAβ after

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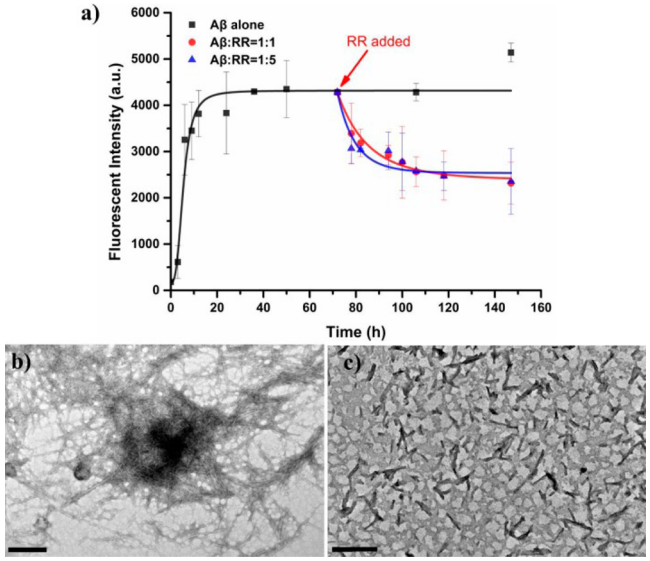

Figure 1. (a) Monitoring detection of Aβ fibrillation and transformation by RR using a ThT fluorescence assay. The fibrils were incubated with 20 μM Aβ monomer, and then RR was dissolved in Aβ fibril solution with a molar ratio of 1:1 and 1:5, respectively (n = 3). (b, c) Negative-staining TEM images of fAβ and rA$\beta_{1:1}$ after 3 days incubation. Scale bars = 500 nm.

treated with RR was also observed by CD spectrum (Figure S1). TEM images were easy to see that the mature fibrils (Figure 1b) with a length of 1 μm or more, actually transformed into nanorod-like fragments (200−250 nm), after eliminating the possibility of the self-assembling of RR (Figure S2). In addition, the obtained rAβ treated with RR of 1:1 (Figure 1c) and 1:5 (Figure S3) molar ratio (represented by rA$\beta_{1:1}$ and rA$\beta_{1:5}$, respectively) had a similar morphology. Because of the great reduction in length, rAβ may be easier for cellular uptake, and the reduction of β-sheet structure may make it facilitated for enzyme-mediated degradation.[16,17] The transformation effect of Aβ inhibitor on Aβ fibril was also reported by other researchers. Chafekar et al. reported a four KLVFF peptides modified dendrimer (K4) and found that K4 could lead to the disassembly of existing aggregates.[22] Although lacking the direct evidence, most studies are apt to attribute this event to the competitive binding between the inhibitor and Aβ fibril.

It is notable that the width of these rAβ was about 25 nm (Figures 1c and S3), which was greater than Aβ protofibril (~5 nm)[23,24] and Aβ mature fibrils (~13−20 nm).[25,26] Thus, we tend to suppose that the RR not only broke the fibrils into fragments, but also bind with rAβ. Moreover, in electrophoresis and MALDI-TOF mass assay, we did not find the corresponding band or molecular ion peak of Aβ oligomers (data not shown here). All these results indicated that instead of completely transforming the fAβ into protofibrils or soluble oligomers, RR partly broke the fibrils into nanorod-like fragments.

Recent research has illustrated that Aβ monomers, oligomers, and protofibrils can interconvert and keep a balance.[27] Once protofibrils grow into fibrils, the latter are insoluble and cannot easily be disassembled.[28] Compared with Aβ monomer and mature fibrils, oligomers and protofibrils are much more neurotoxic.[29−31] Based on these facts, MTT assay was used to examine the cytotoxicity of rAβ on neuronal PC12 cells.[32] Aβ monomers, fibrils, and transformation solutions with the same concentration were added to the cells respectively and cell viability was determined after 48 h. The results (Figure 2)

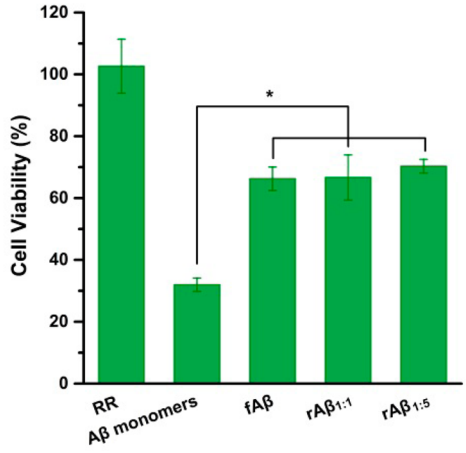

Figure 2. Cytotoxicity of RR and different Aβ species to PC12 cells. The concentration of all samples was 20 μM. Aβ fibrils, rA$\beta_{1:1}$ and rA$\beta_{1:5}$ had a great improvement in cell viability compared with Aβ monomer and they had no significant difference with each other. Cell viability was determined using MTT assay (n = 5). Statistical significance level is expressed by asterisks: *p < 0.01.

demonstrated that rAβ was much less potent to induce cytotoxicity than Aβ monomer. And the similar toxicity of rAβ and fAβ indicated the structural homologue of them. It is also corresponding with our pervious hypothesis.

To further investigate whether the transformation of fAβ could facilitate its cellular uptake, fAβ and rAβ were labeled by FITC, and flow cytometry (FCM) was used to detect the internalized quantity of Aβ assemblies by PC12 cells. FITC-labeled fAβ and rAβ, at a concentration of 2 μM, was cocultured with PC12 cells from 1 to 24 h. The total fluorescence intensity (TFI) of both fibrils and fragments increased significantly during the first 6 h, demonstrating the cellular uptake of rAβ and fAβ. After treated with the Aβ assemblies for 9 h, the TFI of rAβ and fAβ in the cellular fraction decreased, and the TFI in PC12 treated with fAβ, rA$\beta_{1:1}$, and rA$\beta_{1:5}$ showed a similar variation tendency (Figures 3a and S4). Therefore, we chose the maximum TFI point (6 h) to examine the effect of transformation on the cellular uptake

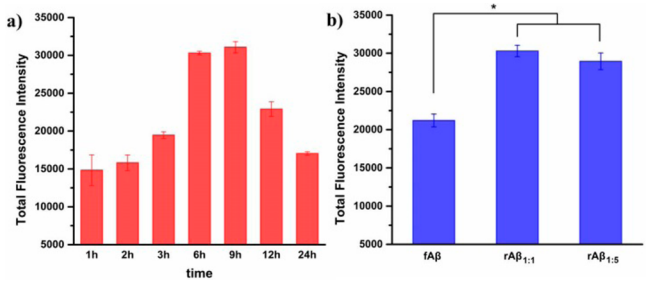

Figure 3. Cellular uptakes of fAβ, rA$\beta_{1:1}$, and rA$\beta_{1:5}$ in PC12 cells. The concentrations of all samples were 2 μM. (a) Total fluorescence intensity of FITC-labeled rA$\beta_{1:1}$ after incubation for 1, 2, 3, 6, 9, 12, and 24 h, respectively. (b) Total fluorescence intensity of FITC-labeled fAβ, rA$\beta_{1:1}$, and rA$\beta_{1:5}$ in cells measured by FCM after incubation for 9 h. Data points shown are the mean values ± SD from three independent experiments. Statistical significance level is expressed by asterisks: *p < 0.01.

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(Figure 3b). As we expected, the cellular uptake of rA$\beta_{1:1}$ was approximately 1.46 times higher than fAβ ($p < 0.01$), suggesting that the great decrease in size may facilitate cellular internalization. And due to the similar particle shape and size, rA$\beta_{1:1}$ and rA$\beta_{1:5}$ did not show a statistical significant difference.

The decrease of Aβ assemblies in PC12 cells perhaps attributed to two reasons: degradation in lysosome or exocytosis by the cells. So confocal microscopy was used to test the intracellular distribution of rAβ and fAβ in cells, and trail the fate after their internalization. PC12 cells were exposed to FITC-labeled rAβ for 3 h and Lysotracker Red was used to visualize lysosomes. It could be seen in Figure 4 that the green

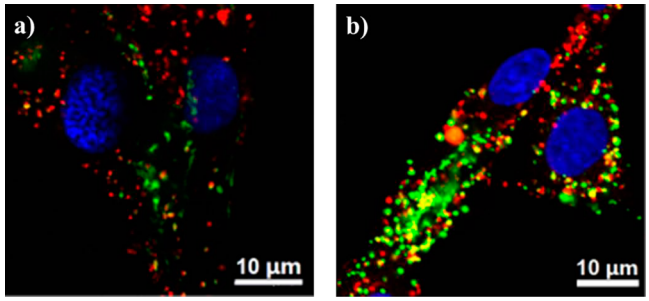

Figure 4. CLSM images of FITC-labeled (a) fAβ and (b) rA$\beta_{1:1}$ (green) with a concentration of 2 μM in PC12 cells after incubation for 3 h. Lysosome (red) was indicated by LysoTracker Red. Cell nuclei (blue) were labeled with DAPI. There was more rA$\beta_{1:1}$ in cells and rA$\beta_{1:1}$ was colocalized with the signal of LysoTracker (yellow) compared with fAβ. Scale bars = 10 μm.

fluorescence in the cell culture with rAβ was obviously stronger than that of fAβ, consistent with the previous FCM result. What's more, we could find lots of colocalization areas (yellow) of rAβ (green) and lysosome (red) in Figure 4b, indicating rAβ was colocalized in lysosomes and possible to be degraded in PC12 cells. This phenomenon could not be obviously observed in fAβ-treated cells, and that maybe because the small quantity of fAβ in cells or the possible exocytosis by cells to clear it.[17]

According to previous research, Aβ is degraded by a large set of proteases with diverse characteristics.[33] Among them, cathepsin B (CatB), a cysteine protease of the papain superfamily, degrades peptides and proteins that enter the endosome/lysosomal system by endocytosis or phagocytosis.[34] It is associated with amyloid plaques in AD brains[33] and it can effectively cleave Aβ42, generating C-terminally truncated Aβ peptides that are less amyloidogenic under cell-free condition.[35] Thus, CatB was chosen to carry out our in vitro degradation of rAβ and fAβ in the following experiments to explore the possibility of the degradation of rAβ after cellular uptake.

After incubating CatB and Aβ monomer/assemblies for 2 h, Aβ were proteolytic cleaved into diverse segments by CatB, which was detected by MALDI-TOF mass spectrometry, and the degraded segments of rAβ were corresponding with those of Aβ monomer and fAβ (Figure 5). From the degradation products of Aβ monomer, it could be seen that CatB has a high activity at Lys_{16}-Leu_{17}, Leu_{17}-Val_{18}, Gly_{33}-Leu_{34}, and Val_{36}-Gly_{37}. It is a favorable property, since Aβ_{16-20} and Aβ_{31-40} are the major hydrophobic regions for the aggregation of Aβ,[36] these C-terminally truncated peptides (Aβ_{1-36}, Aβ_{1-33}, Aβ_{1-17}, and Aβ_{1-16}) will show less fibrillogenic capacity than full-length Aβ_{40}. What's more, the degradation products of rAβ have a relative higher quantity of Aβ_{40}, and there are more C-

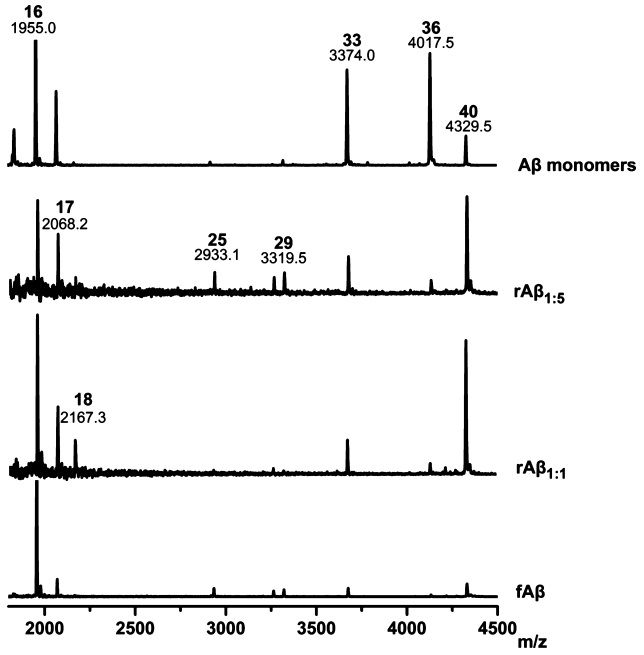

Figure 5. MALDI-TOF mass spectrometry of the cleavage of fresh Aβ monomer, fAβ, rA$\beta_{1:1}$, and rA$\beta_{1:5}$ by pure CatB (0.5 μg/mL) at pH 6.0 (25 mM MES buffer) for 2 h.

terminally truncated peptides in the degradation products of rA$\beta_{1:5}$ than that of rA$\beta_{1:1}$. We suppose this may because of the looser structure of rAβ than fAβs'. This less compact structure of rAβ (especially rA$\beta_{1:5}$) made it easier for the cleavage of CatB, and even made it possible for CatB disaggregating the monomers from rAβ. In addition, the degradation products of fAβ were more difficultly detected than monomers and rAβs, suggesting that the compact intermolecular parallel β-sheets structure of fAβ made the most cleavage-active sites inaccessible to enzymolysis. One exception is that the quantity of Aβ_{1-16} segments seemed higher than other peaks in the degradation products of fAβ. This may come from the cleavage directly from the fibrils, since Aβ_{1-16} is near to the N-terminal of Aβ, which have a random-roil structure. Once an Aβ molecule in fibrils was cleaved at Lys_{16}-Leu_{17}, the Aβ_{1-16} segment was easier to separate and the Aβ_{17-40} segment might remain in the fibril. Altogether, the fact that rAβ were highly susceptible to degrade as compared with fibrils (Figure 5), suggests the promising ability of RR to transform fAβ and facilitate Aβ fibrils degradation in lysosome after cellular internalization.

In summary, an antiamyloidogenic peptide RR was used to transform the mature Aβ fibril into 250-nanometers-long nanorod-like fragments. These fragments were less cytotoxic than amyloidogenic Aβ intermediates and the variance in shape greatly facilitated their cellular internalization. Their colocalization with lysosome after endocytosis and degradation by lysosomal enzyme in vitro greatly indicated the possibility of successfully clearing rAβ in a cell-participated and enzyme-mediated pathway. Thus, our research findings provided a potential method to decrease the accumulation of Aβ from its fibril formation. Further structural studies at high resolution, such as NMR and docking calculations,[37,38] are still needed to fully understand the role of RR in this transformation process. Altogether, these results, integrated with our previous work,[19] indicated that the decapeptide RR could both inhibit the

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aggregations of Aβ and disaggregate the mature Aβ fibril, which makes it a promising modulating molecule for AD treatment.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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