

Comparison of Activity against A β Aggregation between RR and LPFFD*

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Abstract Here, we introduce the design of the structure of RR (RYYAAFFARR), an inhibitor of A β aggregation, using molecular docking, and compare the inhibitory ability between RR and LPFFD, which interacts with A β mainly depending on hydrophobic interaction. Our results showed that RR which can target multiple regions of A β with multiple weak interactions is better than those that only target a single region with the single driving force mainly.

Keywords: Molecular docking; Multiple weak interactions; Affinity; Inhibitory ability.

INTRODUCTION

Amyloid β peptide (A β) is a physiologically important active substance, fibrillization of which leads to Alzheimer's disease (AD). The aggregation of the A β peptide into oligomers or fibrils is considered to be a key process associated with the progression of AD^[1–3]. A main strategy for AD is to target A β , by modulating its aggregation or facilitating its β -sheet aggregates removal from the brain. Therefore, it is essential to design efficient and high affinity ligands for A β .

The combinatorial library strategy was the traditional method for screening ligands, which was tedious and time-consuming however. In recent years, new ligand screening methods have been developed based on the molecular interactions, such as surface plasma resonance (SPR), isothermal titration microcalorimetric (ITC) and molecular simulation screening methods. Recently, molecular simulation^[4] has been extensively used to analyze the intermolecular interactions and screen out new ligands. Molecular docking derives its efficacy from the assumption that a ligand can associate with a macromolecule with predefined binding sites, and the interaction affinity between the ligand and the binding sites is reflected by the calculated energy. The method of molecular docking has been used to screen out new effective ligands, evaluate the character of molecules, study the intermolecular interactions in receptor-ligand binding and interpret their binding modes of interaction^[5]. It's worth mentioning that in our previous work, molecular docking was successfully used in screening out the most effective ligand of affinity adsorbents, and the reliability of the method was also verified by the experiments^[6, 7].

In some literatures, the full A β molecule is divided into five sub-regions: the N-terminus^[8, 9], GAG-binding sites^[10], hydrophobic core^[11, 12], "salt bridge" region^[13–15], and the C-terminus^[16, 17]. Many studies targeted the hydrophobic region of A β (A β _{16–20}, KLVFF) and designed various inhibitors against A β aggregation mainly based on the hydrophobic interaction, such as KLVFF^[18] and LPFFD^[19, 20]. In this study, we expanded the target region of A β from A β _{16–20} to A β _{11–23} (EVHHQKLVFFDAED), which contains three important sub-regions of

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$A\beta$: GAG-binding sites, hydrophobic core, and “salt bridge” region. The expanded target region could offer more interaction sites, such as the hydrophobic, electrostatic interactions and hydrogen bond. Based on the structure of target region, a decapeptide inhibitor RYAAFFARR (RR) with high binding affinity for $A\beta$ was designed using computer simulation quickly and efficiently. In previous study, we demonstrated that as an inhibitor, RR exhibited an extreme stronger binding affinity for $A\beta_{1-40}$ ($K_D = 1.10 \mu\text{mol/L}$) than LPFFD ($K_D = 1.56 \text{ mmol/L}$) and inhibited the aggregation of $A\beta_{1-40}$ effectively. And here we are very curious about the comparison of the inhibitory ability between RR and LPFFD, which is a well-known effective inhibitor of $A\beta$ aggregation. The inhibitory ability of these inhibitors was evaluated with thioflavin T (ThT) fluorescence, transmission electron microscopy (TEM) and a cell viability assay. This study aims at comparing the inhibitory ability between RR and LPFFD and highlighting the importance of multiple weak interactions in the designing of inhibitors of $A\beta$ aggregation.

EXPERIMENTAL

Materials

$A\beta_{1-40}$ peptide (95.52%), $A\beta_{11-23}$ (Ac-EVHHQKLFFFAED-NH₂) (98%), RR (Ac-RYAAFFARR-NH₂) (98%), and LPFFD (99.16%) were purchased from GL Biochem (Shanghai) Ltd. 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, 99%), tungstophosphoric acid and thioflavin T (ThT, 75%) were purchased from Heowns (China). The other reagents were commercially available and used without purification.

Molecular Docking

The structure of $A\beta_{11-23}$ (EVHHQKLFFFAED) was extracted from the crystal structure of full-length $A\beta_{1-42}$ with PDB code 1IYT in the Protein Data Bank (PDB) in our simulations and docking experiments. The model of ligands in the docking was constructed for molecular docking study. Represented molecular structures of the ligands were listed in Fig. 1. During docking, the interaction sites on the ligands didn't change, just varying the spacer between the sites in the peptide sequence. All the structures of ligands were generated on Silicon Graphic Indio workstation using Sybyl 6.91 software package. The sketched molecular structures of ligands were first

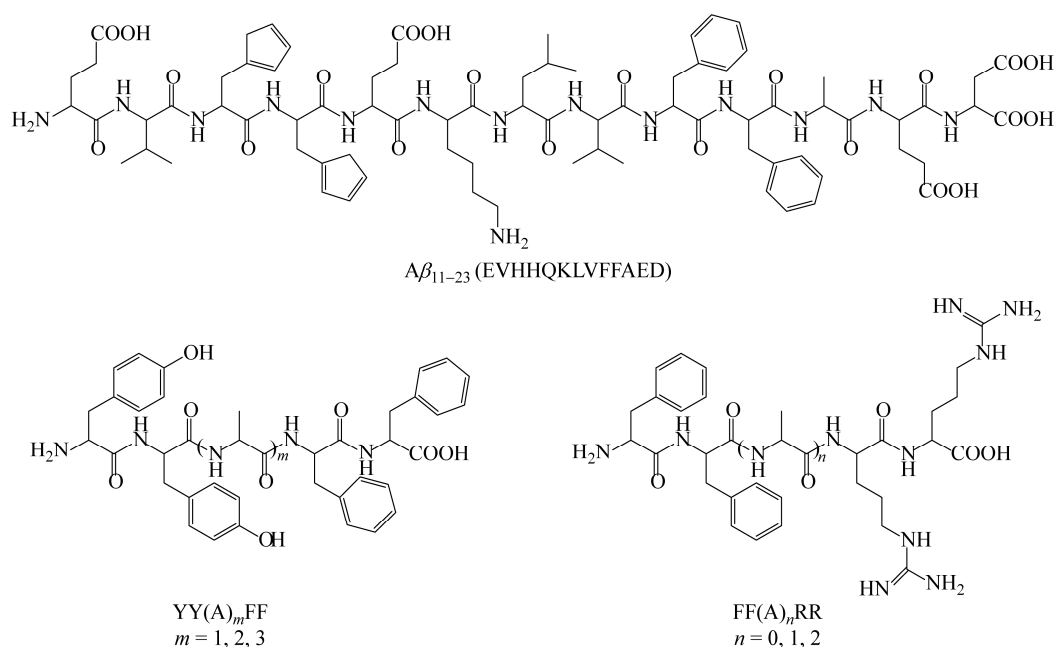


Fig. 1 Chemical structure of $A\beta_{11-23}$ and the ligands: $YY(A)_mFF$, $FF(A)_nRR$

optimized by Powell's gradient algorithm method using Tripos force field with a distance-dependent dielectric constant until a terminating gradient of $0.21 \text{ kJ}\cdot\text{mol}^{-1}\cdot\text{nm}^{-1}$ was reached. The Gasteiger-Huckel charge was chosen. Then the obtained conformation of the ligand was refined by the simulated annealing method for 100 cycles. During annealing, the temperature of the molecule varied from 100 K to 1000 K for 1000 fs. The conformation with the lowest energy for the ligand molecule was selected and minimized again subsequently^[20]. In the experiments, five modes with the lowest energy were chosen for every ligand to dock to $A\beta_{11-23}$ to obtain the average binding energy.

Molecular docking was used to characterize the interactions of ligands with $A\beta_{11-23}$. $A\beta_{11-23}$ docking to the ligands with five different conformations was performed using the Autodock 4.0 program with five different initial binding modes for each ligand. And the lowest energy conformation of ligands was chosen as the initial molecular conformation for docking. The average of the five separately obtained lowest energies was used to evaluate the affinity between $A\beta_{11-23}$ and ligands. During the docking, the model of the ligands was allowed to be flexible and $A\beta_{11-23}$ was fixed. All the computations were carried out with grid box spacing 0.0375 nm centered at the interaction region. Grid search was performed using the Lamarckian genetic algorithm. A total of 500 docking runs was conducted to find the best conformation and ligand orientation based on the binding energy (E_{binding})^[21, 22], which was deduced from the following expressions. And the lowest binding energy of each docking was analysed.

$$E_{\text{binding}} = E_{\text{complex}} - E_{A\beta} - E_{RR}$$

where E_{complex} was the binding energy of $A\beta_{11-23}$ with RR, $E_{A\beta}$, E_{RR} represented the energy of $A\beta_{11-23}$ and RR, respectively.

Thioflavin T (ThT) Fluorescence Assay

The $A\beta_{1-40}$ fibrillization in the presence and absence of inhibitors was monitored by ThT fluorescence assay, which was carried out by an F-7000 fluorescence spectrophotometer (Hitachi, Japan). The concentration of ThT store solution was $500 \mu\text{mol/L}$, and then the ThT solution was further diluted into phosphate buffer (pH 7.4) to reach a final concentration of $10 \mu\text{mol/L}$. A volume of $100 \mu\text{L}$ of $A\beta_{1-40}$ with or without the inhibitor was put into $900 \mu\text{L}$ ThT solution, and then the mixture was blended briefly. All ThT fluorescence measurements were operated using a $1 \text{ cm} \times 1 \text{ cm}$ quartz cuvette (excitation at 440 nm and emission at 485 nm). Each sample was measured three times, and then the data were averaged.

Transmission Electron Microscopy

All experiments were performed by using TEM (FEI, Tecnai G² F20, USA). To detect the effect of the peptide inhibitors on $A\beta_{1-40}$ aggregation, TEM was used to observe the peptide morphology. Samples were prepared by placing a $15 \mu\text{L}$ droplet of sample solution on a carbon support Cu coated grid for 90 s, then the excess fluid was wicked away. Subsequently, the samples were negatively stained with 1% tungstophosphoric acid for 90 s, the excess fluid was removed and the grids were placed in a vacuum drier and dried at room temperature.

Cell Viability Assays

Highly differentiated rat pheochromocytoma (PC-12) cells were cultured in a medium of 90% Dulbecco Modified Eagle Medium (DMEM) and 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 . For cell viability assays, cells were plated in 96-well plates at a density of 3×10^4 cells per well and maintained for 24 h.

To evaluate the biological cytotoxicity of the RR itself, peptide solutions were prepared by dissolving the RR peptide in deionized water and diluting into DMEM medium to yield concentrations of $10\text{--}160 \mu\text{mol/L}$. 24 h after plating of cells, $150 \mu\text{L}$ of the RR solutions in DMEM were added into the 96-well plate. The cells were then incubated for 18 h with the inhibitor RR at 37°C . Cell viability was determined by the thiazolyl-bule-tetrazolium-bromide (MTT) assay. Briefly, $20 \mu\text{L}$ stock MTT (5 mg/mL) mixed with $100 \mu\text{L}$ DMEM medium was added into the 96-well plate, and the plate was incubated at 37°C . Four hours later, $120 \mu\text{L}$ DMSO was added after the medium-MTT solution was removed. Finally, absorbance intensity at 490 nm was measured with

an ELISA reader. At least three independent experiments with five replicates were carried out, and the results were averaged.

To test the inhibitory effect of the peptide inhibitor RR on $A\beta_{1-40}$ -induced toxicity, solutions of $A\beta_{1-40}$ and RR at three different ratios (1:1, 1:2, 1:4) were prepared, and $A\beta_{1-40}$ alone was regarded as the control experiments. In this part of experiment, the solutions of $A\beta_{1-40}$ with or without RR were pre-incubated for 12 h in DMEM. Cell viability was determined by the MTT assay as described above.

RESULTS AND DISCUSSION

In this paper, based on the structure of the target, we designed a series of peptide ligands $YY(A)_mFF$ and $FF(A)_nRR$, which could offer multiple weak interaction sites, including hydrophobic and electrostatic interactions and hydrogen bonding. The alanines in the ligands played a role of spacer between the interaction sites. Then the molecular docking method was used to screen the most efficient ligand. During the docking process, the functional amino acids in the ligand sequence were kept constant, but the length of the spacers (m , n) between interaction sites was varied. Because of the limitation of the Autodock, we first study the spacer length (m) between YY and FF, and then determine the spacer length (n) between FF and RR.

Designing of the Structure of RR

After the conformation and energy of the $YY(A)_mFF$ ($m = 1, 2, 3$) molecules were optimized, five different modes of $YY(A)_mFF$ were fitted to the $A\beta_{11-23}$ molecule. Then the fitted molecular pairs containing a ligand and $A\beta_{11-23}$ were subjected to 500 cycles blind docking. The average of the five separately obtained lowest energies was used to evaluate the binding affinity between the ligand molecules and $A\beta_{11-23}$. The docking results are listed in Fig. 2. From Fig. 2, it could be found that the docking of YYAFF, YYAAFF and YYAAAFF with $A\beta_{11-23}$ gave negative binding energies in all cases, which indicated that these three structures could form stable complexes with $A\beta_{11-23}$. Moreover, the hexapeptide YYAAFF showed the lowest binding energy for $A\beta_{11-23}$ compared with YYAFF and YYAAAFF, which indicated that the higher affinity between YYAAFF and $A\beta_{11-23}$.

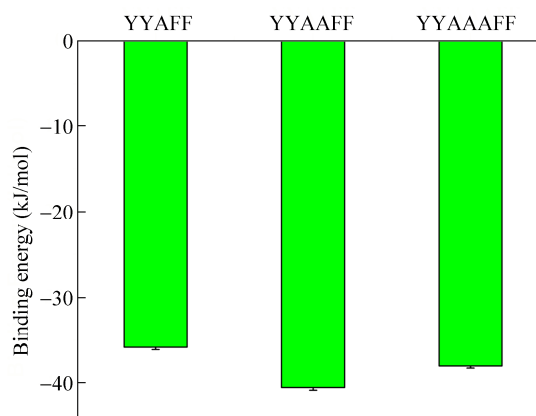


Fig. 2 The binding free energy between $YY(A)_mFF$ and $A\beta_{11-23}$ ($m=1, 2, 3$)

In addition, the molecular docking method was employed to investigate possible interaction patterns in a three-dimensional space. Figure 3 shows two best conformations of YYAAFF docked to $A\beta_{11-23}$. It is confirmed that the hydrophobic interaction occurs when the hydrophobic motif of the ligand closes to the hydrophobic domain of the target molecule. From Fig. 3, it was clear that after the docking process, the benzene ring of phenylalanine in the YYAAFF sequence tended to locate around the Phe 19 and Phe 20 of $A\beta_{11-23}$. What's more, the active phenol-OH groups of YYAAFF effectively formed hydrogen bonds with the imidazole group of the His13 or His14. However neither YYAFF nor YYAAAFF could form the hydrophobic interaction and hydrogen bond simultaneously (data not shown), which was in agreement with the result of binding free energy.

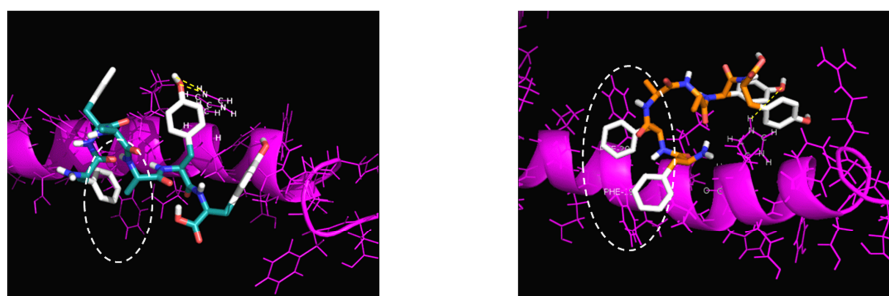


Fig. 3 The best binding modes of YYAAFF to $A\beta_{11-23}$ after docking process. The hydrophobic interaction between YYAAFF and $A\beta_{11-23}$ was denoted with the white dotted line circle, and the yellow dotted line represented the hydrogen bonding between the phenol-OH of YYAAFF and the histidine of $A\beta_{11-23}$.

The distance between the hydrogen bond and hydrophobic interaction in the ligand have been determined ($m = 2$), it was necessary to confirm the spacer between the hydrophobic interaction and the electrostatic interaction. In the same way, molecular docking was used for screening of the candidate ligand by comparing the binding energies between different ligands and $A\beta_{11-23}$. Considering the structure of $A\beta_{11-23}$, we designed three ligands with three different spacers FFRR, FFARR and FFAARR. As shown in Fig. 4, the docking of FFRR, FFARR and FFAARR with $A\beta_{11-23}$ all gave negative binding energies, indicating that these three ligands could form stable complexes with $A\beta_{11-23}$. It also could be found that the pentapeptide FFARR ($n = 1$) showed the highest binding affinity to $A\beta_{11-23}$ with the lowest binding energy for $A\beta_{11-23}$ compared with FFRR and FFAARR.

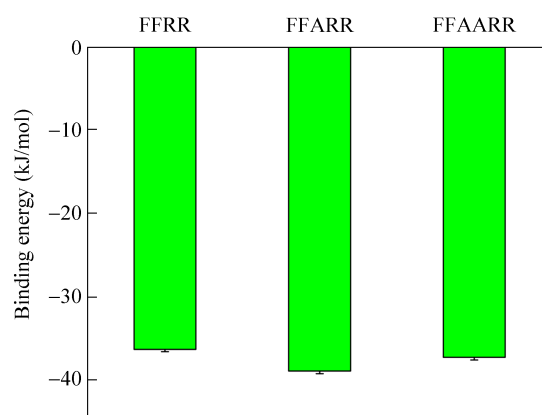


Fig. 4 The binding free energy between FF(A)_nRR and $A\beta_{11-23}$ ($n = 0, 1, 2$)

It was well known that computer simulation could offer the imaginable interaction pattern between ligands and receptors. Figure 5 displayed the two best modes of FFARR binding to $A\beta_{11-23}$. In Fig. 5, the phenylalanine of FFARR got close to the Phe19 or Phe20 of $A\beta_{11-23}$, which might be driven by the hydrophobic interaction. The arginine of the ligand formed electrostatic interaction with the Asp23 of $A\beta_{11-23}$, which helped the ligand and the $A\beta_{11-23}$ molecule to achieve better performance. According to the screening by molecular docking, YYAAFF and FFARR were probably effective structures for the target peptide $A\beta_{11-23}$. So the spaces between the hydrogen bond and hydrophobic interaction sites, the hydrophobic interaction and electrostatic interaction sites were confirmed. Then after integrating, a decapeptide RYYAAFFARR (RR) was obtained, and the arginine in the N-terminus of RR was introduced to avoid the aggregation of RR itself.

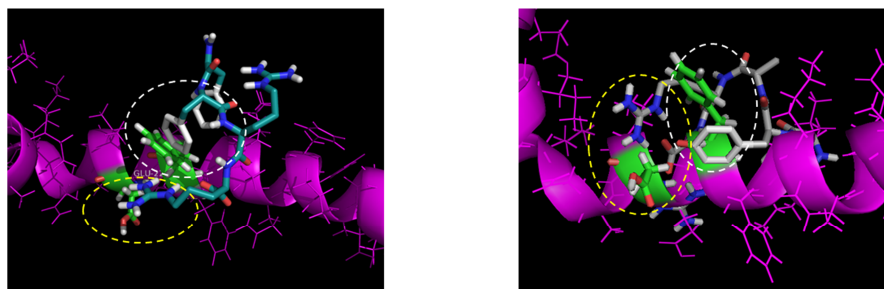


Fig. 5 The best binding modes of FFARR to $A\beta_{11-23}$ after docking process. The hydrophobic interaction between FFARR and $A\beta_{11-23}$ was denoted with the white dotted line circle, and the yellow dotted line circle represented the electrostatic interaction between the arginine of FFARR and the Asp23 of $A\beta_{11-23}$.

RR is a Ligand with Higher Affinity for $A\beta$ than LPFFD

For an effective inhibitor, there is a relationship between its affinity for $A\beta$ and its inhibition of $A\beta$ aggregation and toxicity. In our design, the inhibitor RR has multiple interaction sites, and in our previous study^[23], the SPR experiment revealed that RR interacted specifically with the immobilized target peptide, the $A\beta_{1-40}$ monomer with a dissociation constant (K_D) of 1.10 $\mu\text{mol/L}$. We also tested the affinity of LPFFD for the $A\beta_{1-40}$ monomer, and the K_D value was 0.156 mmol/L. Thus, the SPR assays showed that RR had a higher binding affinity for $A\beta_{1-40}$ than LPFFD, which preliminarily verified the advantage of the multiple weak interactions between RR and $A\beta_{1-40}$.

Viet^[24] provided theoretical support for the correlation between the binding affinity and the inhibitory ability: stronger binding between the inhibitor and $A\beta$ correlates with more effective inhibition. Cairo^[25] also demonstrated with SPR assays that ligands with greater affinity for $A\beta$ effectively altered its aggregation and inhibited its cell toxicity. Our results show that RR binds to the $A\beta_{1-40}$ molecule with stronger binding affinity, which was two orders of magnitude higher than the binding affinity of LPFFD. Therefore, we examined whether RR inhibits the aggregation of $A\beta_{1-40}$ more effectively than LPFFD in the next section.

RR is a More Potent Inhibitor of $A\beta$ Aggregation than LPFFD

$A\beta_{1-40}$ was prepared as described in the Experimental Section. Then $A\beta$ was cultured in the presence of RR or LPFFD in different ratios. In order to compare the effects of RR and LPFFD on $A\beta$ aggregation, ThT fluorescence, transmission electron microscopy (TEM), and a cell viability assay were conducted.

ThT fluorescence assays results

We used ThT fluorescence assays to investigate the effects of RR and LPFFD on $A\beta$ aggregation based on the specific binding of the ThT dye to β -sheet structures^[26]. The results (Fig. 6) of the ThT binding assay of $A\beta_{1-40}$, in the absence or presence of different concentrations of RR and LPFFD, showed that RR strongly and effectively inhibited β -sheet aggregation in $A\beta_{1-40}$ in a dose-dependent manner. However, LPFFD slightly influenced the aggregation of $A\beta_{1-40}$. In particular, equimolar mixtures of $A\beta_{1-40}$ and RR reduced the relative change in the ThT fluorescent signal by nearly 53%. Incubation of $A\beta_{1-40}$ and RR in a molar ratio of 1:5 resulted in a 91% reduction in the fluorescent signal, which did not increase during incubation for 10 days, while LPFFD reduced the fluorescent signal by just 37.5% at the same concentration. The ThT fluorescent assay indicated that RR is a more potent inhibitor of $A\beta_{1-40}$ aggregation than LPFFD. In our study, RR had higher affinity for the $A\beta_{1-40}$ monomer and inhibited the aggregation of $A\beta_{1-40}$ much more effectively than LPFFD. So the results of the ThT fluorescence experiments correspond to the relative affinities measured with SPR assays.

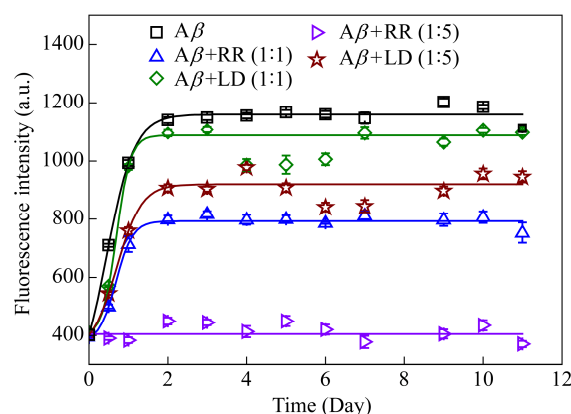


Fig. 6 β -Sheet structure of $A\beta_{1-40}$ (20 $\mu\text{mol/L}$) monitored using ThT fluorescence in the absence and presence of different concentrations of RR and LPFFD (The samples were incubated at 37 $^{\circ}\text{C}$ for 10 days with agitation.)

TEM results

To confirm the better inhibitory effect of RR on $A\beta_{1-40}$ aggregation than LPFFD, TEM was used to study the morphology of the aggregates that formed in the absence and presence of RR or LPFFD. When $A\beta_{1-40}$ was incubated alone for 7 days, the sample formed mature fibrils (Fig. 7a), which were approximately 1–10 nm wide and tens to hundreds of nanometers long. In the presence of 1–5 molar equivalents of RR, the morphology of $A\beta_{1-40}$ changed and the fibril content decreased dramatically (Figs. 7b–7c). When the molar ratio ($A\beta$:RR) was 1:5, the aggregation of $A\beta_{1-40}$ was strongly inhibited by RR, and only amorphous aggregates were visible but without any ordered fibrillar aggregates on the entire copper grid (Fig. 7c). While in the mixtures of $A\beta_{1-40}$ and LPFFD under the same conditions for 7 days, short and thick fibrils were evident (Fig. 7d). The TEM images of the morphology of the mixture of $A\beta$ /LPFFD (1:5) confirmed that LPFFD had less effect on $A\beta_{1-40}$ aggregation than RR, as suggested by the ThT fluorescence assay.

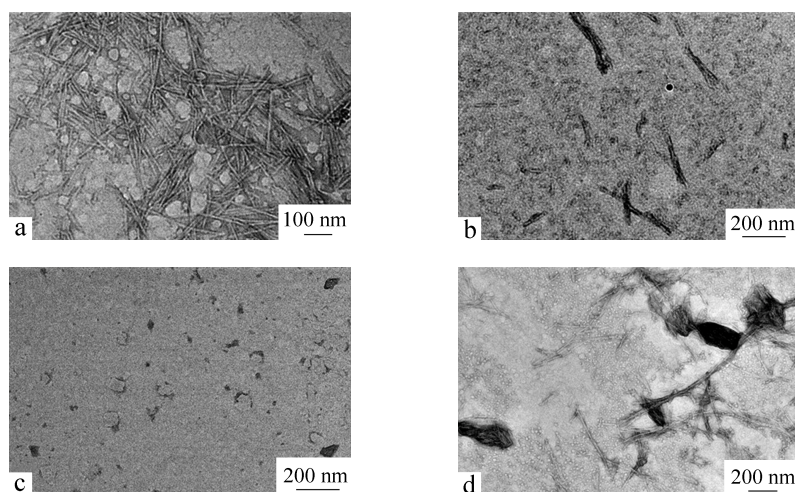


Fig. 7 TEM images of $A\beta_{1-40}$ (20 $\mu\text{mol/L}$) incubated with RR or LPFFD for seven days: (a) $A\beta_{1-40}$ alone, (b) RR 20 $\mu\text{mol/L}$, (c) RR 100 $\mu\text{mol/L}$ and (d) LPFFD 100 $\mu\text{mol/L}$

Cell viability assays results

The toxicity of $A\beta$ lies in its soluble oligomeric forms and is related to its self-assembly. Therefore, we compared the effect of RR and LPFFD as a peptide inhibitor of $A\beta_{1-40}$ -induced toxicity by measuring cell viability.

Figure 8 shows that both RR and LPFFD protected PC-12 cells from $A\beta_{1-40}$ -induced toxicity, but to different extents. Cell viability after $A\beta_{1-40}$ (20 $\mu\text{mol/L}$) treatment was approximately 65% that of the control, whereas incubation of $A\beta_{1-40}$ with RR strikingly enhanced cell viability. Furthermore, RR in a molar ratio of 1:1 ($A\beta$:RR) was sufficient to significantly prevent $A\beta_{1-40}$ -induced toxicity and $A\beta_{1-40}$ aggregation. The viability of PC-12 cells increased to about 95% when $A\beta_{1-40}$ was incubated with RR in a molar ratio of 1:5 ($A\beta_{1-40}$:RR), which was almost equal to the control. Figure 8 also shows that RR inhibited $A\beta_{1-40}$ -induced cytotoxicity to a greater extent than LPFFD at the same concentration. Because $A\beta$ neurotoxicity is related to the formation of the β -sheet structure^[27], the MTT assay results suggest that RR is a more potent inhibitor of $A\beta_{1-40}$ than LPFFD, supporting the results of the ThT fluorescence assays.

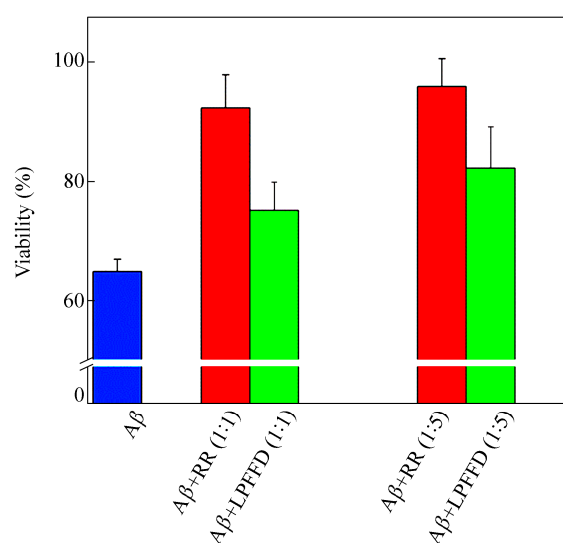


Fig. 8 Viability of PC12 cells treated with $A\beta_{1-40}$ (20 $\mu\text{mol/L}$) alone or with different concentrations of RR or LPFFD for 18 h. Cell viability was measured with MTT assays and is shown as a percentage of the untreated cells.

CONCLUSIONS

In this paper, based on the chemical structure of the expanded target peptide $A\beta_{11-23}$, a series of ligands bearing the same binding sites but different spacers between different binding sites were designed. To obtain the best ligand, molecular docking method was used to screen out the ligands with the appropriate spacer by calculating the binding energies and comparing the space conformations. As a result, a decapeptide RR possessing multiple weak interaction (hydrophobic and electrostatic interactions and hydrogen bond) sites was screened out as the best ligand for $A\beta_{11-23}$ among the designed ligands. Our previous study about SPR experiments indicated that RR exhibited a strong binding affinity for $A\beta_{1-40}$ that was two orders of magnitude higher than that of LPFFD, which mainly formed single hydrophobic interaction with $A\beta$. In this paper, we found that RR more potently inhibits the aggregation and fibrillation of $A\beta_{1-40}$ than LPFFD according to the results of a ThT fluorescence assay and TEM. RR also markedly reduces the cytotoxicity induced by $A\beta_{1-40}$ self-assembly more effectively, according to an MTT assay. The results of this study suggest that utilizing the synergy of multiple weak interactions is a valid and promising strategy for the design and development of novel effective inhibitors of AD.

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