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The binding of resveratrol to monomer and fibril amyloid beta

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ABSTRACT

As currently understood, Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is driven by the aggregation of amyloid beta (Aβ) protein. It has been shown that resveratrol (RES) may attenuate amyloid β peptide-induced toxicity, promote Aβ clearance and reduce senile plaques. However, it remains to be determined whether RES could interact directly with Aβ. The aim of the present study was to examine the direct binding of RES to monomer and fibril Aβ. Using surface plasmon resonance (SPR) and proton nuclear magnetic resonance (^{1}H NMR), our results identified the direct binding of RES to Aβ. The ability of RES to bind to both fibril and monomer Aβ(1–40 and 1–42) was further analyzed by SPR. The binding response of RES to fAβ(1–42) was higher than that to monomer Aβ(1–42), whereas the binding response of RES to fAβ(1–40) was lower than that to monomer Aβ(1–40). The K_D of RES for fibril Aβ(1–40 or 1–42) was higher than that for the corresponding monomer Aβ. Compared to the control compound Congo red (CR), the binding responses of RES to monomer Aβ(1–42) and Aβ(1–40) were stronger, but binding to fibril Aβ(1–42) was weaker, and the K_Ds of RES with both monomer and fibril Aβ(1–40) and Aβ(1–42) were higher than that of CR. When Aβ(1–40 or 1–42) was co-incubated with RES (50 μM), the thioflavin T fluorescence of the mixture was weakened, and the number and length of amyloid fibrils were decreased. Furthermore, the results of staining in consecutive brain slices from AD patients showed that RES (10^{-4} M) could stain senile plaques. These results indicated that RES could bind directly to Aβ in different states, which may provide new insight into the protective properties of RES against AD.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia (Walsh and Selkoe, 2004; Mattson, 2004). The presence of many senile plaques and neurofibrillary tangles in the brain are two hallmarks of AD pathology. According to the amyloid cascade hypothesis, the aggregation of amyloid beta (Aβ) is a primary driving force in the pathogenesis of the disease (Hardy and Selkoe 2002; Korczyn, 2008). It was found that the elevation of the deposition of Aβ peptides in the cortex, particularly those ending at amino acid 42 (Aβ1–42), is an early and invariant feature of all forms of AD. The accumulation of Aβ could initiate a series of downstream neurotoxic events, resulting in neuronal dysfunction in AD patients (Korczyn, 2008). These findings have strongly motivated the search for therapies targeting the aggregation of Aβ (Amijee and Scopes, 2009; Carter et al., 2010).

Resveratrol (trans-3,5,4-trihydroxy-trans-stilbene, RES) is a polyphenol that is found mainly in grapes and red wine and has diverse beneficial biological and pharmacological activities, including anti-inflammatory (Zhu et al., 2008) and antioxidant (Robb et al., 2008) effects. Recently, a number of studies have focused on the neuroprotective effects of RES against AD (Sahebkar, 2010; Marambaud et al., 2005; Karuppagounder et al., 2009), demonstrating that this compound could attenuate amyloid β peptide-induced toxicity (Huang et al., 2011), promote Aβ clearance (Marambaud et al., 2005) and reduce senile plaques (Karuppagounder et al., 2009) in cell or AD mouse models through its potent antioxidant activity (Robb et al., 2008). To date, only few reports have systematically described the effects of RES on Aβ self-aggregation (Feng et al., 2009), and it remains to be determined whether RES could interact directly with Aβ.

In the present study, we explored the direct binding of RES to monomer and fibril Aβ, as determined by surface plasmon resonance (SPR), thioflavin T (ThT) fluorometric analysis, proton nuclear magnetic resonance (^{1}H-NMR), and atomic force microscopy (AFM) in vitro. Furthermore, we investigated whether this compound was capable of labeling senile plaques in a comparison with thioflavin S (ThS) staining in consecutive brain slices from AD patients.

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2. Materials and methods

2.1. Chemicals and reagents

Resveratrol (trans-3,5,4′-trihydroxy-trans-stilbene, approximately 99% purity, RES), dimethylsulfoxide (DMSO), thioflavin T (ThT), thioflavin S (ThS) and Congo red (CR) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The structures of RES and CR are shown in Fig. 1. Aβ(1–42) and Aβ(1–40) were purchased from Anaspec Inc. (San Jose, CA, USA). All other reagents used in the experiments were of analytical grade.

Hippocampal samples from AD patients were obtained within the framework of the rapid autopsy program of The Netherlands Brain Bank (coordinator, Dr. Ingle). Permission was obtained for brain autopsy and the use of the tissue and patient clinical information for research purposes.

2.2. Preparation of Aβ and fibril Aβ (fAβ) solutions

The Aβ and fAβ solutions were prepared as described previously (Hirohata et al., 2007), with some modifications. Dry Aβ(1–42) was dissolved by brief vortexing in ice-cold 0.1% ammonia solution at a concentration of approximately 250 μM (1.1 mg/mL) in a 4 °C room. To remove possible fibrils, the solution (0.8 mL) was transferred to polycarbonate tubes and ultracentrifuged at $10^5 g$ for 3 h at 4 °C, and the upper three-quarter fraction was collected by careful aspiration and stored at −80 °C before assaying [fresh Aβ(1–42) solution]. Aβ(1–40) was dissolved by brief vortexing in ice-cold distilled water at approximately 500 μM (2.2 mg/mL) in a 4 °C room and stored at −80 °C before assaying [fresh Aβ(1–40) solution].

Fibril Aβ(1–42) [fAβ(1–42)] was formed from the fresh Aβ(1–42) solution. The reaction was performed in an Eppendorf tube in 950 μL and contained 25 μM Aβ(1–42), 50 mM phosphate buffer (pH 7.5), and 100 mM NaCl. After brief vortexing, the mixture was incubated at 37 °C for 24 h to allow polymerization. The reaction tubes were not agitated during the reaction. After incubation, the mixture was centrifuged at 4 °C for 3 h at 1.5×10^4 rpm using a high-speed refrigerated microcentrifuge. The pellet was resuspended in 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0.05% NaN_3 in an Eppendorf tube, sonicated on ice with 15 intermittent pulses (pulse 0.6 s, interval 0.4 s, output level 2) from an ultrasonic disruptor equipped with a microtip, and stored at 4 °C before assaying. Another type of fAβ [fAβ(1–40)] was formed from the fresh Aβ(1–40) solution. The reaction was performed in a total of 600 μL and contained 50 μM Aβ(1–40), 50 mM phosphate buffer (pH 7.5), and 100 mM NaCl. After incubation at 37 °C for 24 h, the mixture was centrifuged, resuspended, and sonicated as described above and stored at 4 °C before assaying.

2.3. Affinity analysis of resveratrol to Aβ/fAβ by surface plasmon resonance (SPR)

2.3.1. Immobilization of Aβ/fAβ on the sensor chip of a Biacore biosensor

A BIAcore 3000 (Biacore Inc., Piscataway, NJ, USA) equipped with four flow cell channels on one sensor chip was used for real-time binding studies. Two carboxymethylated dextran 5 (CM5) sensor chips were used in this experiment: one for Aβ(1–40) and Aβ(1–42), and the other for fAβ(1–40) and fAβ(1–42).

Fresh Aβ(1–40) was immobilized on the CM5 chip in the second channel, and the first channel was used as a reference (without immobilization of Aβ(1–40)). Aβ(1–42) was immobilized in the fourth channel, and the third channel was used as a reference. The carboxymethyl dextran surface was activated using 70 μL of a mixture of 0.4 M N-ethyl-N′-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) at an injection rate of 10 μL/min, followed by the injection of Aβ(1–40) into channel 2 or Aβ(1–42) into channel 4 (70 μL of Aβ was diluted with 600 μL of pH 4.0 sodium acetate buffer and injected at a rate of 5 μL/min). After Aβ immobilization, the remaining activated groups were blocked with 70 μL of 1 M ethanolamine (pH 9.0). The reference cell was prepared by the above-described amine coupling without the addition of Aβ. The running buffer solution contained 50 mM PB, 100 mM NaCl, 0.3 mM EDTA, and 0.05% Tween 20, and the washing solvent contained 1% DMSO, pH 7.4.

fAβ(1–42) and fAβ(1–40) stored at 4 °C were centrifuged at 4 °C for 2 h at 16,000*g*. fAβ precipitated completely, and the pellet was resuspended in water, sonicated, diluted with 10 mM sodium acetate (pH 4.0), and immobilized immediately on the other CM5 sensor chip by amine coupling.

2.3.2. Immobilization of bovine serum albumin (BSA) on the sensor chip of a Biacore biosensor

To investigate whether RES binding was specific to Aβ proteins, bovine serum albumin (BSA) was immobilized on a CM5 chip with a blank channel as a reference under the same condition as Aβ immobilization.

2.4. Affinity and kinetics analysis

After immobilization, solutions of different compounds with different concentrations were injected into the cell, and binding was observed. Each binding experiment was repeated three times, and the reaction was performed at 25 °C using the running buffer mentioned above. Compounds (CR, ThS, and RES) dissolved in DMSO were diluted with running buffer to a final concentration of 0–100 μM and injected over the reference and Aβ/fAβ-immobilized flow cells at a flow rate of 30 μL/min. The surface of the sensor chip immobilized with Aβ or fAβ was regenerated with a mixture of 0.025% sodium lauryl sulfate (SDS) and 25 mM sodium hydroxide (NaOH). Data from the reference cell without Aβ or fAβ immobilization were subtracted from raw data. The running buffer injection using the same method was also subtracted as a double reference (Yan et al., 2007).

2.5. Thioflavin T (ThT) fluorescence assay

fAβ(1–42) and fAβ(1–40) were quantitated using ThT on a fluorescence spectrophotometer (BMG Polarstar Optima). The reaction mixtures (200 μL) containing 50 μM monomeric Aβ(1–42) or Aβ(1–40), 50 mM PB buffer (pH 7.5), 100 mM NaCl, and 0 μM or 50 μM RES were incubated at 37 °C for 24 h in a black microfluo-

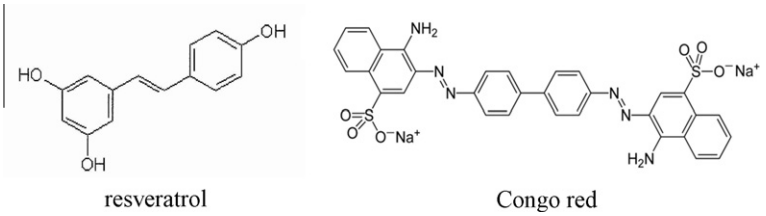

Fig. 1. Structures of the compounds examined in this study.

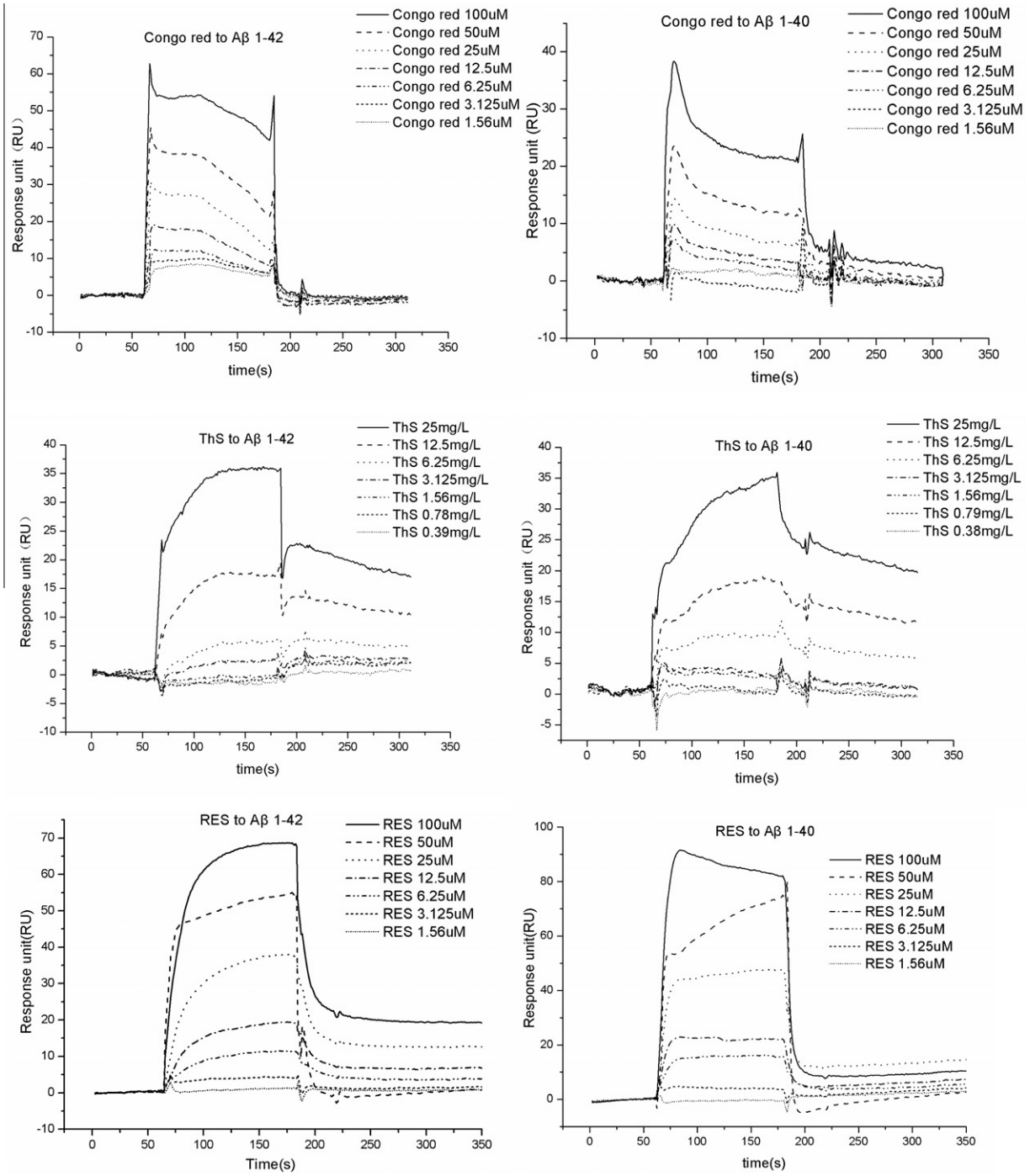

Fig. 2. Analysis of the affinities of CR, ThS, and RES for the Aβ monomer by SPR. Congo red (CR), thioflavin S (ThS) and resveratrol (RES) were dissolved in DMSO and diluted with running buffer [50 mM phosphate (pH 7.5), 100 mM NaCl, 3 mM EDTA, 0.005% Tween 20, and 1% DMSO]. The solutions with different concentration were injected over the reference and Aβ(1–42) (left) or Aβ(1–40) (right)-immobilized flow cells. Three experiments were performed at each concentration.

rometer cell. The optimal fluorescence was measured at excitation and emission wavelengths of 445 and 490 nm, respectively, with a reaction mixture containing 5 μM ThT and 50 mM glycine-NaOH buffer (pH 8.5).

2.6. ^{1}H NMR spectroscopy

Samples for ^{1}H NMR were dissolved in aqueous PBS with 10% $2H_2O$ (v/v) added. Samples containing Aβ(1–40) or Aβ(1–42) were run at 0.3 mM (Barnham et al., 2008). RES dissolved in DMSO was diluted with $2H_2O$ to 50 μM and incubated with Aβ(1–40) or Aβ(1–42) at 37 °C for 2 h. ^{1}H NMR spectra were recorded on Bruker DMX-600 and AMX-500 spectrometers equipped with cryo-probes at 293 K.

2.7. Atomic force microscopy (AFM)

The reaction mixtures were the same as that used in the ThT fluorescence assay. After incubation at 37 °C for 24 h, 10 μL of the solution was spotted onto freshly cleaved mica adsorption surfaces for 5 min and washed briefly with water to reduce the background contaminants. AFM was carried out in air using an SPA-400

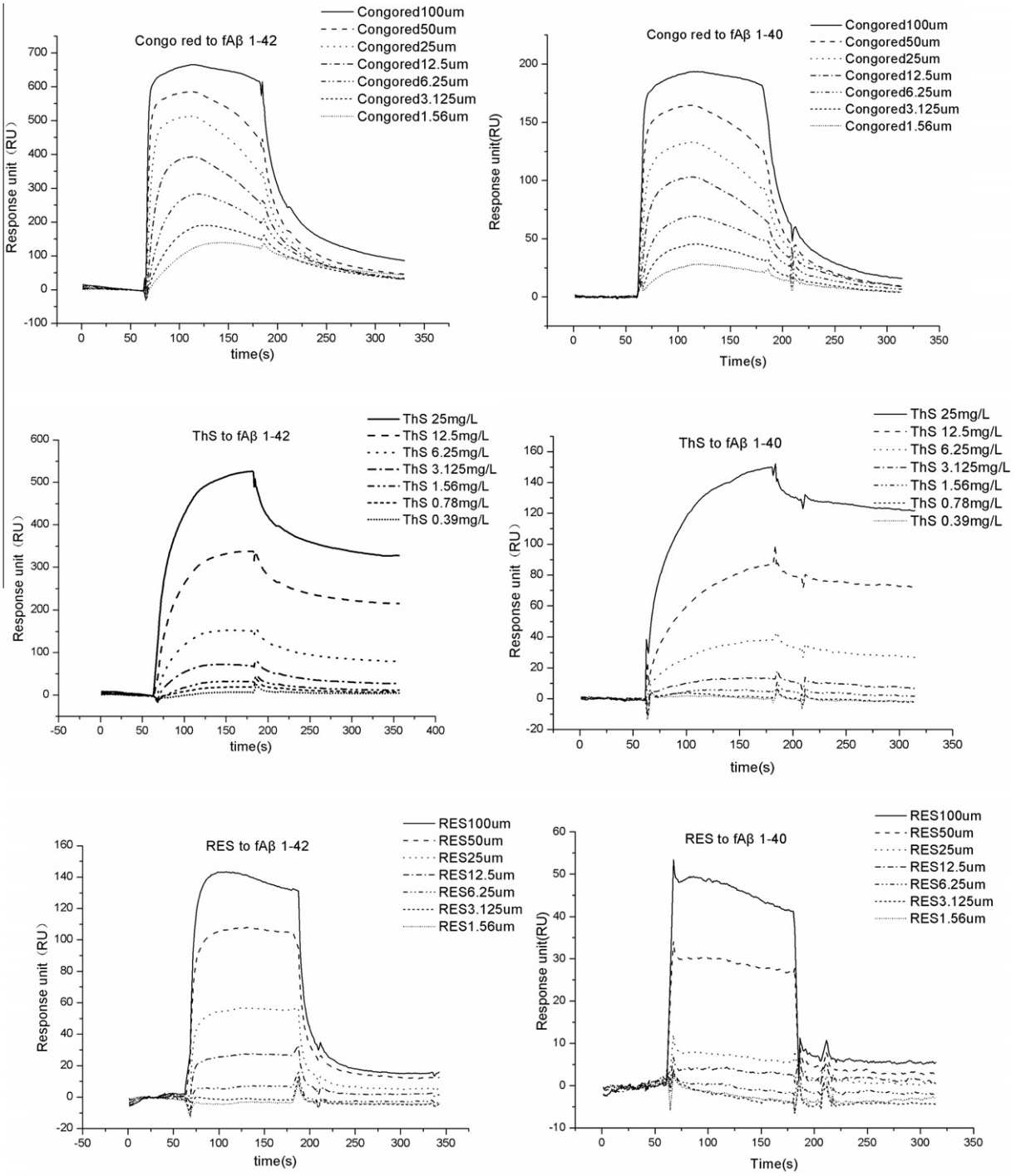

Fig. 3. Analysis of the affinities of CR, ThS, and RES for Aβ fibrils by SPR. Congo red (CR), thioflavin S (ThS) and resveratrol (RES) were dissolved in DMSO and diluted with running buffer [50 mM phosphate (pH 7.5), 100 mM NaCl, 3 mM EDTA, 0.005% Tween 20, and 1% DMSO]. The solutions with different concentration were injected over the reference and fAβ(1–42) (left) or fAβ(1–40) (right) immobilized flow cells. Three experiments were performed at each concentration.

scanning probe microscope controlled by an SPI 3800F probe station. The cantilevers were rectangular and had a force contact of 12 N/m and a resonance frequency of 128 kHz at tip scan rates from 0.5 to 1 Hz. At least four regions of the mica surface were examined to confirm the homogeneity of the structures throughout the sample.

2.8. Consecutive brain section staining with RES

Postmortem brain tissues from an autopsy-confirmed case of AD were used. A total of 6-μm paraffin sections were cut with a Leica microtome (Leica RM 2135) and processed for staining according to previously described methods (Hou et al., 2006; Duan et al., 2010). Briefly, the histological sections were deparaffinized, rehydrated and washed three times in phosphate-buffered saline (PBS, pH 7.4, 0.01 M) for 5 min. After being rinsed in 0.05% potassium permanganate for 20 min and washed three times in PBS for 5 min, the sections were treated with a solution of 0.2% oxalic acid and 0.2% potassium metabisulfite for 1 min and stained for 10 min in a solution of 0.2% ThS or 1×10^{-4} M RES for 1 h. To ensure the full solubilization of the compound, 50% ethanol was used. Subsequently, the sections were washed in tap water and differen-

Table 1
Binding Response, Dissociation Constant (K_D) of Congo red and RES to monomer and fibril Aβ by SPR.

Group		Binding response[a] (RU/Da)	K_D[b] (μM)
Aβ1–42	CR	0.076 ± 0.003	30.567 ± 0.208
	RES	0.275 ± 0.015**	72.700 ± 11.547**
	ThS	n.d.[c]	n.d.[c]
Aβ1–40	CR	0.052 ± 0.013	42.043 ± 8.478
	RES	0.412 ± 0.001**	147.333 ± 46.177*
	ThS	n.d.[c]	n.d.[c]
fAβ1–42	CR	0.996 ± 0.005##	13.733 ± 3.029#
	RES	0.727 ± 0.011**▲	176.667 ± 37.447**▲
	ThS	n.d.[c]	n.d.[c]
fAβ1–40	CR	0.271 ± 0.018##	12.757 ± 3.325##
	RES	0.264 ± 0.006▲▲	724.000 ± 193.098*▲
	ThS	n.d.[c]	n.d.[c]

[a] Binding response value was calculated from the R_{eq} value divided by the molecular weight at a concertration of 100 μM.
[b] K_D values were determined by steady state analysis.
[c] n.d. not determined because its structure and molecular weight were undetermined.
* $P < 0.05$, compared to the binding response or KD of CR to corresponding state of Aβ
** $P < 0.01$, compared to the binding response or KD of CR to corresponding state of Aβ.
$P < 0.01$, compared to the binding response or KD of CR to corresponding monomer Aβ.
▲ $P < 0.05$, compared to the binding response or KD of RES to corresponding monomer Aβ.
▲▲ $P < 0.01$, compared to the binding response or KD of RES to corresponding monomer Aβ.

tiated for 15 min in 50% ethanol. Then, sections were rinsed off with PBS, aqueous mounted and imaged with epifluorescence. Fluorescent sections were viewed using a Leica AF6000 LX microscope with GFP (for ThS) or CFP/YFP (for RES) filters.

2.9. Data analysis

The binding response, which is an index of the interaction of a compound with molecules on a biosensor chip, was obtained from the equilibrium response (R_{eq}) value in the sensorgram divided by the molecular weight. The dissociation constant (K_D) was calculated from data at doses ranging from 1.56 to 100 μM by steady-state analysis using BIAevaluation software (version 4.1). The results of the ThT fluorescence intensity were expressed as the mean ± standard error of the mean (S.E.M.), and between-group differences were analyzed using the Student's *t* test.

3. Results

3.1. RES could bind to both monomer and fibril Aβ(1–40) and Aβ(1–42)

As shown in Fig. 2 and Fig. 3, distinct association and dissociation reactions were observed after the addition of freshly prepared CR, ThS or RES to immobilized monomer and fibril Aβ(1–40) or Aβ(1–42), indicating that all the three compounds could react with the immobilized Aβ monomer or fibril. The responses of CR and RES reached equilibrium (R_{eq}) quickly and returned to baseline rapidly after dissociation. The response of CR to fibril Aβ(1–42) showed the strongest signal. However, ThS neither reached the R_{eq} state within the association phase nor returned to baseline in the dissociation phase, demonstrating slight dissociation and firm binding to Aβ.

The binding response was calculated as the R_{eq} value divided by the molecular weight. As shown in Table 1, the binding response of CR to fibril Aβ (0.271 ± 0.018 to fAβ(1–40) and 0.996 ± 0.005 to fAβ(1–42)) was higher than that to the corresponding monomer Aβ (0.052 ± 0.013 to Aβ(1–40) and 0.076 ± 0.003 to Aβ(1–42)), but the binding response of RES to fAβ(1–42) (0.727 ± 0.011) was higher than that to monomer Aβ(1–42) (0.275 ± 0.015), whereas the binding response of RES to fAβ(1–40) (0.264 ± 0.006) was lower than that to monomer Aβ(1–40) (0.412 ± 0.001). Compared to CR, the binding response of RES was higher to monomer Aβ(1–42) and monomer Aβ(1–40) ($P < 0.01$) but lower to fibril Aβ(1–42) ($P < 0.01$).

The dose–response curve for CR and RES appeared to be monophasic, and it reached a saturation level at higher concentrations. The dissociation constant (K_D) was calculated using steady-state analysis, and the K_D of CR to fibril Aβ (12.757 ± 3.325 to fAβ(1–40) and 13.733 ± 3.029 to fAβ(1–42)) was significantly lower than that to the corresponding monomer Aβ (42.043 ± 8.478 to Aβ(1–

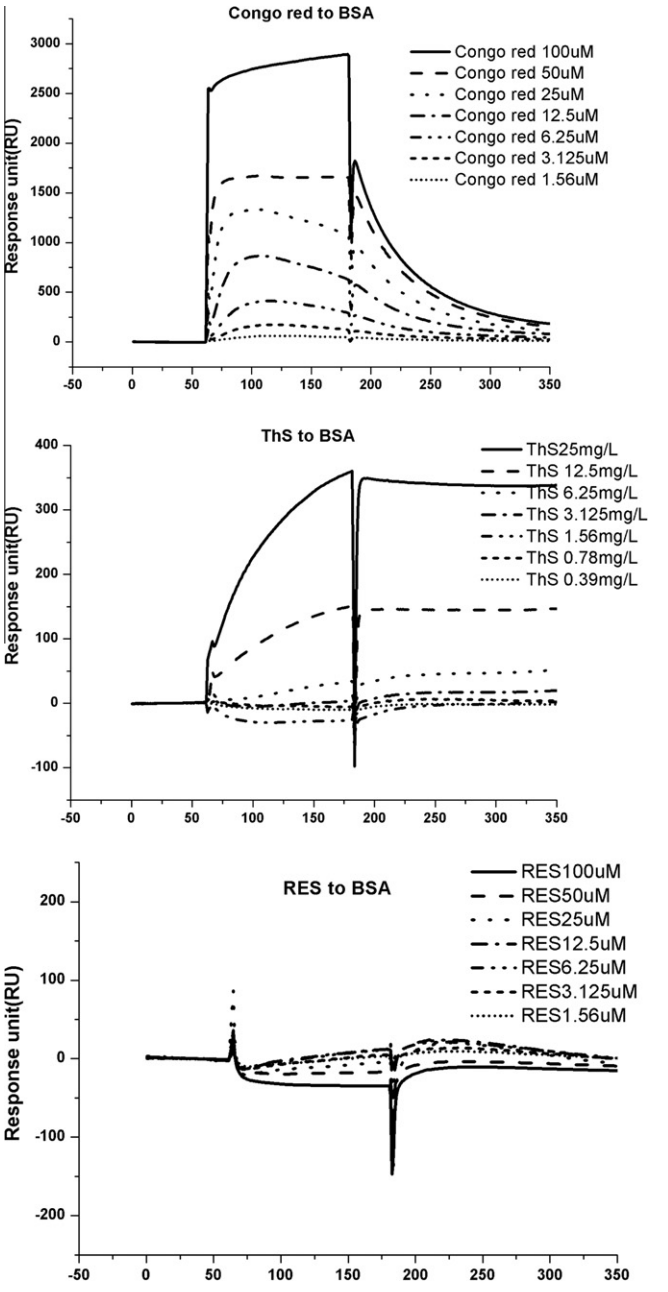

Fig. 4. Analysis of the affinities of CR, ThS, and RES for BSA by SPR. Congo red (CR), thioflavin S (ThS) and resveratrol (RES) were dissolved in DMSO and diluted with running buffer [50 mM phosphate (pH 7.5), 100 mM NaCl, 3 mM EDTA, 0.005% Tween 20, and 1% DMSO]. The solutions with different concentration were injected over the reference and BSA-immobilized flow cells. Three experiments were performed at each concentration.

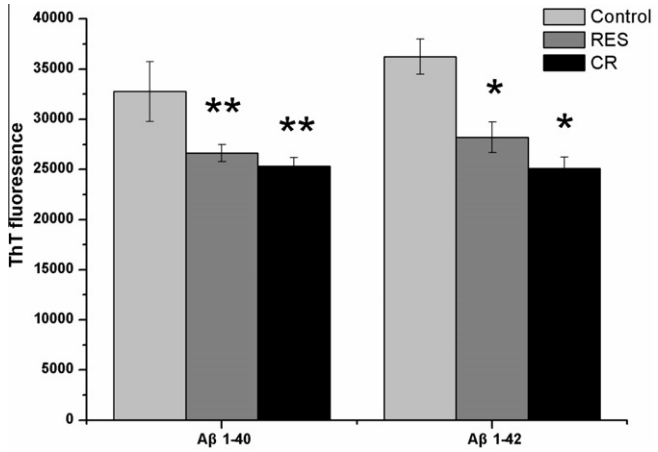

Fig. 5. The effect of RES (50 μM) co-incubation with Aβ(1–42) or Aβ(1–40) on the intensity of ThT fluorescence. ($\bar{X} + s, n = 4$) $^{*}P < 0.05$, $^{**}P < 0.01$, vs. Control).

40) and 30.567 ± 0.208 to Aβ(1–42)). However, the K_D of RES to fibril Aβ (724.000 ± 193.098 to fAβ(1–40) and 176.667 ± 37.447 to fAβ(1–42)) was higher than that to the corresponding monomer Aβ (147.333 ± 46.177 to Aβ(1–40) and 72.700 ± 11.547 to Aβ(1–42)). The K_Ds of RES to both monomer and fibril Aβ(1–40) and Aβ(1–42) were higher than that of CR (Table 1).

Fig. 4 shows the SPR sensorgrams of CR, ThS and RES to immobilized BSA. Distinct association and dissociation reactions were also observed after the addition of freshly prepared CR or ThS but not RES.

3.2. RES co-incubation with monomer Aβ(1–40) or Aβ(1–42) decreased the fluorescence intensity of ThT

After incubation of Aβ(1–42) or Aβ(1–40) with fresh CR or RES at pH 7.5 and 37 °C for 24 h, ThT fluorescence was significantly reduced compared with controls (Fig. 5).

3.3. NMR spectroscopy demonstrated the direct binding between RES and A peptides

To confirm the binding between RES and Aβ peptides, we monitored their interaction with ^{1}H NMR. Two peaks of approximately 1.05 ppm and 1.72 ppm emerged (Fig. 6B) after the addition of RES to the Aβ(1–42) monomer, while the addition of RES to Aβ(1–40) caused the disappearance of two peaks at approximately 1.17 ppm (Fig. 6D). The strong perturbation of both the Aβ(1–42) and Aβ(1–40) monomer proved the direct binding between RES and Aβ peptides.

3.4. AFM image

After incubation at 37 °C and pH 7.5 for 24 h, typical fibrillar Aβ1–42 and Aβ1–40 were observed by AFM (Fig. 7A and C). RES (50 μm) co-incubation with monomer A markedly reduced the number and length of amyloid fibrils formed (Fig. 7B and D), but small amorphous aggregates and short, sheared fibrils were observed (Fig. 7B and D).

3.5. RES could bind to Aβ plaques in brain sections of AD

Senile plaques in hippocampal tissue sections were clearly stained by RES, which was confirmed by staining adjacent sections with ThS. As shown in Fig. 8, both the senile plaques and tangles were stained by ThS (Fig. 8A and C). RES demonstrated a specific affinity for Aβ plaques (Fig. 8B and D), and relatively lower background staining was observed.

4. Discussion

In the present study, we unequivocally demonstrated that RES could directly bind to both monomeric and fibrillar amyloid structures. The binding response of RES to fAβ(1–42) was higher than that to monomer Aβ(1–42), whereas the binding response of RES

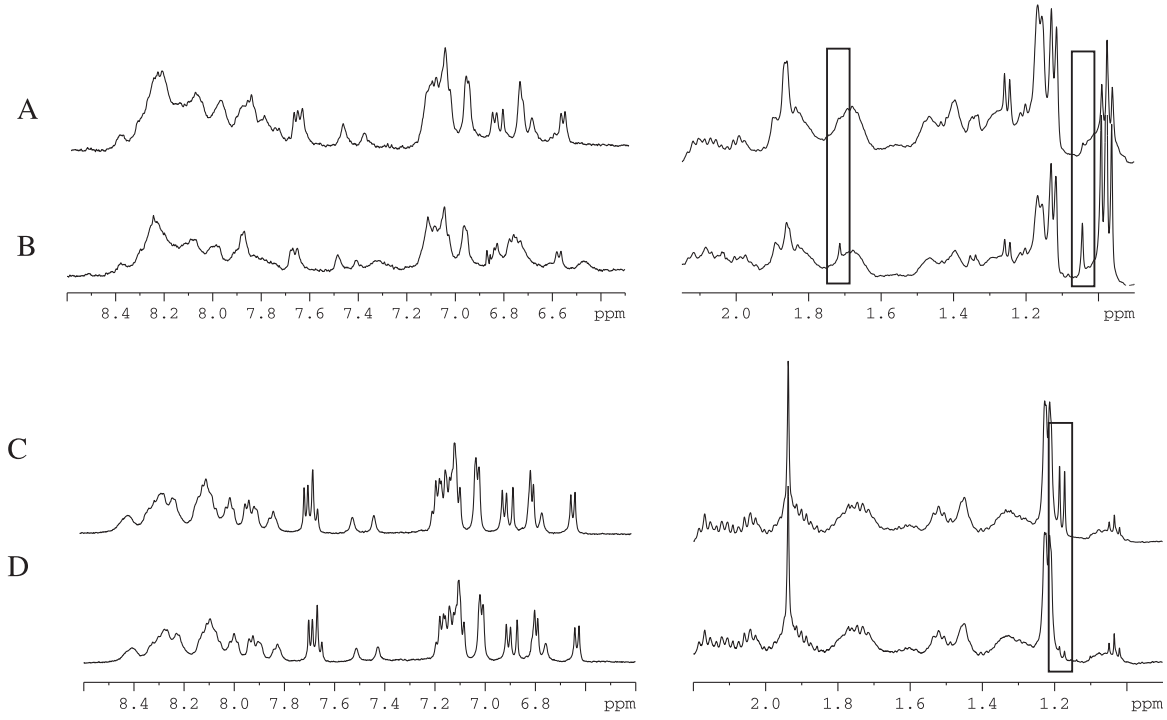

Fig. 6. The effect of RES (50 μM) on the self-aggregation of Aβ(1–42) or Aβ(1–40) by ^{1}H NMR. A: Aβ(1–42) monomer (300 μM), 37 °C, 2 h. B: Aβ(1–42) (300 μM) + RES (50 μM), 37 °C, 2 h. C: Aβ(1–40) monomer (300 μM), 37 °C, 2 h. D: Aβ(1–40) (300 μM)+RES (50 μM), 37 °C, 2 h.

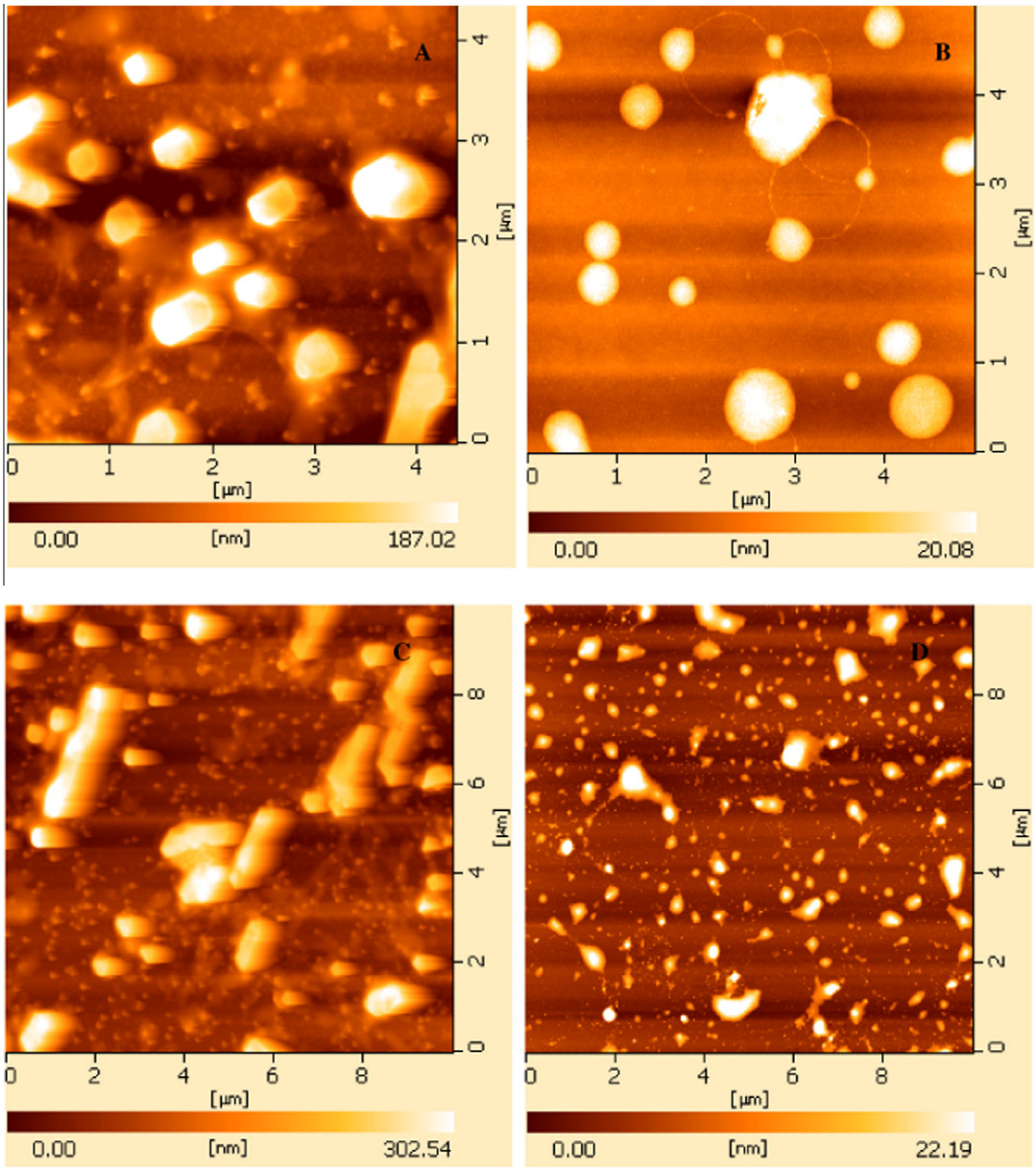

Fig. 7. Atomic force microscopic assessment of the effects of RES (50 μM) on the formation of fAβ(1–42) or fAβ(1–40) from fresh Aβ(1–42) or Aβ(1–40). The reaction mixture contained 50 μM Aβ(1–42) or Aβ(1–40), 50 mM phosphate buffer (pH 7.5), 100 mM NaCl, and 0 (A and C) or 50 μM RES (B and D) and was incubated at 37 °C for 24 h. A: Aβ(1–42) monomer (50 μM), 37 °C, 24 h. B: Aβ(1–42) (50 μM) + RES (50 μM), 37 °C, 24 h. C: Aβ(1–40) monomer (50 μM), 37 °C, 24 h. D: Aβ(1–40) (50 μM) + RES (50 μM), 37 °C, 24 h.

to fAβ(1–40) was lower than that to monomer Aβ(1–40), and the K_D of RES to fibril Aβ was higher than that to the corresponding monomeric Aβ. Compared to CR, the binding response of RES was higher for monomer Aβ(1–42) and monomer Aβ(1–40) but lower for fibril Aβ(1–42), and the K_D of RES to both monomer and fibril Aβ(1–40) and Aβ(1–42) was higher. The results of ThT fluorescence detection and AFM showed that RES (50 μM) co-incubation with monomer Aβ(1–42) or A(1–40) markedly reduced amyloid fibrillation, and the direct interaction of RES with Aβ(1–42) or Aβ(1–40) was demonstrated by ^{1}H NMR. Moreover, the present results showed that RES could stain senile plaques in brain slices from an AD patient.

Substantial biochemical and genetic evidences indicate that Aβ(1–40) and Aβ(1–42) are the dominating Aβ isoforms in AD brains(Hardy and Selkoe 2002; Portelius et al., 2010). Targeting at either Aβ(1–40) or Aβ(1–42), numerous agents have been tested for their potency in preventing the fibrillization of Aβ in vitro or reducing plaque loads in transgenic mice, and some of these compounds have reached the human clinical trial stage (Jhee and Shiovitz et al., 2003; Salloway et al., 2011). As a potential anti-neurodegenerative compound (Richard et al., 2011), the neuroprotective effects of RES have recently been demonstrated and linked to the Aβ peptide (Lu et al., 2009; Marambaud et al., 2005; Karuppagounder et al., 2009). Compelling evidence showed that RES could attenuate Aβ peptide-induced toxicity (Lu et al., 2009; Marambaud et al., 2005) and promote clearance (Lu et al., 2009; Marambaud et al., 2005), and it could be used as a reagent for the quantitative determination of amyloid fibrils (Ahn et al., 2007). The general affinity of RES for peptides and proteins might be a possible explanation for these observations. However, it was not clear whether RES can directly interact with Aβ, and recent reports suggested that RES does not act through anti-aggregative pathways but via its scavenging properties (Granzotto and Zatta, 2011).

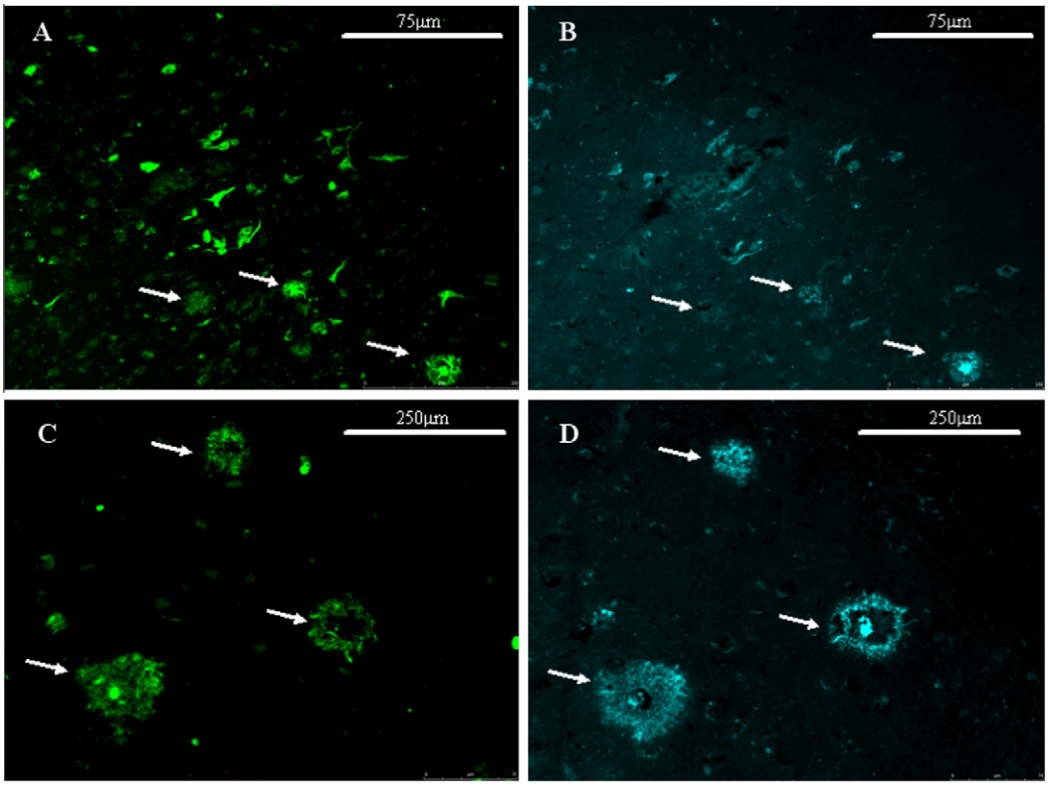

Fig. 8. Representative images of RES and ThS staining in AD human brain slices in vitro. Similar to ThS (B, D), RES stained senile plaques (A, C) in brain slices from AD patients.

However, substantial differences between Aβ (1–40) and Aβ (1–42) with regard to their structure and stability (Picou et al., 2011; Olofsson et al., 2007), aggregation propensity (Riek et al., 2001; Chen and Glabe, 2006; Olofsson et al., 2007), distribution and role in AD pathology (Shao et al. 1999; Gregory and Halliday 2005) make it important to identify whether there are discrepancies of the binding or inhibitory ability for RES to Aβ (1–40) and Aβ (1–42) under same condition. Furthermore, in view of the different charge and hydrodynamic radius between monomer and fibril Aβ (Picou et al., 2011), which could change the fibril assembly rate (Bedrood et al., 2012) and provide different binding site (Petkova et al., 2006), we also investigate the binding or inhibitory ability of RES to both monomer and fibril Aβ in the present study.

CR is a small molecule that specifically reacts with amyloids, and it has been used since the 1920s as the analytical "gold standard" for amyloid characterization. It was reported that CR could bind to fibril Aβ(1–40) in two steps (Pedersen et al., 2010), and hydrogen bonds and hydrophobic, aromatic, and ionic interactions have been assumed to contribute to the binding of CR to amyloid fibrils (Schutz et al., 2011). Consistent with the findings from Maezawa (Maezawa et al., 2008), the results of SPR in the present study showed that CR could bind directly to both monomer and fibril Aβ, with a higher binding response and lower K_D to fibril Aβ than to the corresponding monomer Aβ, indicating the larger binding amounts and higher binding affinity of CR to fibril Aβ compared with monomer Aβ. Similar to CR, RES and ThS could also bind to both monomer and fibril Aβ with different binding characteristics. Compared to CR, both the binding response and the K_D of RES to monomer Aβ(1–42) and monomer Aβ(1–40) were higher, indicating that RES bound in greater amounts but with a lower binding affinity to monomer Aβ than did CR. However, the binding response of RES to fibril Aβ(1–42) was lower and binding to fibril Aβ(1–40) was similar to that of CR, while the K_D of RES to both fibril Aβ(1–40) and Aβ(1–42) was higher than that of CR, indicating that the potency of RES binding to fibril Aβ was weaker than that of CR. Although the mechanism underlying the different binding characteristics of RES and CR remains unknown, it might be partly inferred from the findings that RES could selectively remodel Aβ conformers, including soluble oligomers, fibrillar intermediates, and amyloid fibrils, into an alternative aggregated species that is non-toxic, has a high molecular weight, and is unstructured (Ladiwala et al., 2010). These K_D values, however, were only rough estimations and might be underestimated due to the lack of data for concentrations higher than 100 μM. The K_D of ThS was not precisely calculable because ThS is presumed to be a mixture of compounds formed by methylation and sulfonation of primulin, whose structures and molecular weights have not been determined.

Furthermore, discrepancies of the binding ability of RES to Aβ(1–40) and Aβ(1–42) was found in the present study. The binding response of RES to fAβ(1–42) was higher than that to monomer Aβ(1–42), but the binding response of RES to fAβ(1–40) was lower than that to monomer Aβ(1–40), and the K_D of RES to fibril Aβ was higher than that to the corresponding monomer Aβ. These results indicated again the discriminating features between Aβ(1–40) and Aβ(1–42) monomers. Moreover, this findings highlighted the importance of the consideration of different Aβ segment or state used when interpret the binding or inhibitory ability of compound to Aβ, especially when different results were found.

In addition to Aβ, Congo red has also been shown to bind to the native or partially folded states of several different proteins (Piekarska et al., 2001; Khurana et al., 2001), including BSA (Zhang et al., 2009). Consistent with these results, the SPR sensorgram obtained in the present study indicated that CR could bind directly to BSA. However, there were no typical association and dissociation reactions between RES and immobilized BSA, indicating the different binding specificities of RES and CR. Combined with the purported antioxidant and anti-inflammatory (Zhu et al., 2008) properties of RES, as well as its possible therapeutic effects on AD (Sahebkar, 2010; Karuppagounder et al., 2009; Huang et al., 2011), and lack of adverse effects (Boocock et al., 2007), it is

rational to consider RES as a potential therapeutic compound and diagnostic probe in AD.

The fluorescence of the benzothiazole dye thioflavin T (ThT) is a well-known test for amyloid fibril formation (Sulatskaya et al., 2011, 2012). Although the molecular mechanism of the dye binding to the fibrils is not well established, Krebs (Krebs et al., 2005) reported that the dipole excitation axis of ThT was parallel to the long molecular axis of amyloid fibrils and proposed that ThT binding occurred in "channels" that run along the length of the β-sheet. It was reported that CR could interfere with ThT fluorescence (Buell et al., 2010), consistent with this report, the results of the present study showed that the ThT fluorescence intensity of the CR group was significantly reduced after incubation with monomer A beta. Moreover, the ThT fluorescence intensity was reduced when monomer Aβ(1–40) or Aβ(1–42) was co-incubated with RES, indicating less amyloid fibrillation. Combined with the SPR results in the present study, which showed distinct association and dissociation reactions between RES with monomer and fibril Aβ, it is reasonable to consider that RES could reversibly or noncovalently bind to the β-sheet structure of Aβ. The direct interaction of RES with Aβ(1–40) or Aβ(1–42) was demonstrated by our ^{1}H NMR results.

Using transmission electron microscopy, Feng et al. (2009) demonstrated that co-incubating RES with Aβ(1–42) could dose-dependently inhibit Aβ(1–42) fibril formation and cytotoxicity but could not prevent Aβ(1–42) oligomerization (Feng et al., 2009). It has also been reported that when RES is added, the usual fibrillation pathway of human islet amyloid polypeptide (IAPP), which has similar secondary structure in the fibrillar state and shares amino acid sequence similarity with the ordered region of Aβ, (Jayasinghe and Langen, 2004) is diverted to an off-pathway product consisting of spherical amorphous oligomers with dominant secondary random coil structure (Jiang et al., 2011). In support of this result, RES (50 μM) co-incubation with monomer Aβ (1–42) or Aβ(1–40) markedly reduced the number and length of the amyloid fibrils formed, but small amorphous aggregates and short, sheared fibrils were observed.

To further investigate the ability of RES to bind to the Aβ structure, consecutive brain slices from AD patients were used to determine whether RES could directly stain Aβ plaques (SP), which are extracellular amyloid deposits. It has been identified that ThS could bind to protein aggregates in AD including Aβ and tau protein (Santa-Maria et al., 2006; Siegemund et al., 2006). Consistent with this, both the senile plaques and tangles were stained by ThS in the present study. RES also demonstrated a qualitative specific affinity for Aβ plaques, and relatively lower background staining was observed.

Taken together, the results of the present study show for the first time that RES could bind directly to both monomer and fibril Aβ, which provides a further understanding of the mechanism of action of RES as a possible therapeutic treatment for AD.

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