

## Inhibition of Aggregation of Amyloid $\beta$ 42 by Arginine-Containing Small Compounds

Takayasu KAWASAKI\* and Shunsuke KAMIJO†

*Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan*

Received November 16, 2011; Accepted December 24, 2011; Online Publication, April 7, 2012

[doi:10.1271/bbb.110879]

**Aggregations of proteins are in many cases associated with neurodegenerative diseases such as Alzheimer's (AD). Small compounds capable of inhibiting protein aggregation are expected to be useful for not only in the treatment of disease but also in probing the structures of aggregated proteins. In previous studies using phage display, we found that arginine-rich short peptides consisting of four or seven amino acids bound to soluble 42-residue amyloid  $\beta$  ( $A\beta$ 42) and inhibited globulomer (37/48 kDa oligomer) formation. In the present study, we searched for arginine-containing small molecules using the SciFinder searching service and tested their inhibitory activities against  $A\beta$ 42 aggregation, by sodium dodecyl sulfate (SDS)-PAGE and thioflavine T binding assay. Commercially available Arg-Arg-7-amino-4-trifluoromethylcoumarin was found to exhibit remarkable inhibitory activities to the formation of the globulomer and the fibril of  $A\beta$ 42. This chimera-type tri-peptide is expected to serve as the seed molecule of a potent inhibitor of the  $A\beta$  aggregation process.**

**Key words:** aggregation; Alzheimer's disease; amyloid  $\beta$ ; coumarin; globulomer

The processes by which proteins aggregate into amyloid fibrils are in many cases associated with neurodegenerative disorders.<sup>1)</sup> Amyloid  $\beta$  ( $A\beta$ ) is well-known to aggregate into fibrils, an important hallmark of Alzheimer's disease (AD), and the development of effective medicines to target the aggregation of  $A\beta$  is under way all over the world.<sup>2,3)</sup> It was suggested recently that soluble oligomers of  $A\beta$  as well as the fibrils are toxic to neuronal cells.<sup>4)</sup> Although it is necessary for structural and physiological studies to prepare a homogeneous  $A\beta$  protein *in vitro*, most soluble oligomers are of various molecular sizes (from 10 kDa to 100 kDa). Among the soluble oligomers reported, globulomer (37/48 kDa, apparent mass of two bands on SDS-PAGE gel) can be prepared reproducibly and homogeneously in the presence of sodium dodecyl sulfate (SDS) or fatty acids *in vitro*, and it binds to the hippocampal neurons of rat brain tissue and blocks long-term potentiation at an early stage of AD.<sup>5)</sup> Hillen *et al.* generated a monoclonal antibody against globulomer and suggested the possibility that antibody is effective for therapy in AD.<sup>6)</sup> This strategy is

based on the concept that suppression of function of the resulting toxic oligomers is effective for the reduction of amyloid toxicity. On the other hand, we have focused on forestalling soluble oligomer formation, and have screened compounds that inhibit the production of toxic globulomer.<sup>7,8)</sup> Globulomer is perhaps not an intermediate of  $A\beta$  fibril formation, as indicated by solution NMR analysis.<sup>9,10)</sup> Therefore, at least two kinds of toxic soluble oligomers of  $A\beta$  are present in the brain cells of AD patients, globulomer, and soluble oligomers produced during the course of fibrillation. In order to reduce the toxic  $A\beta$  molecule completely, drugs capable of inhibiting both globulomer and fibril formation are considered to be effective.<sup>11)</sup> Also, such a double-functional compound is expected to be useful for analysis of the aggregation mechanism of  $A\beta$ .

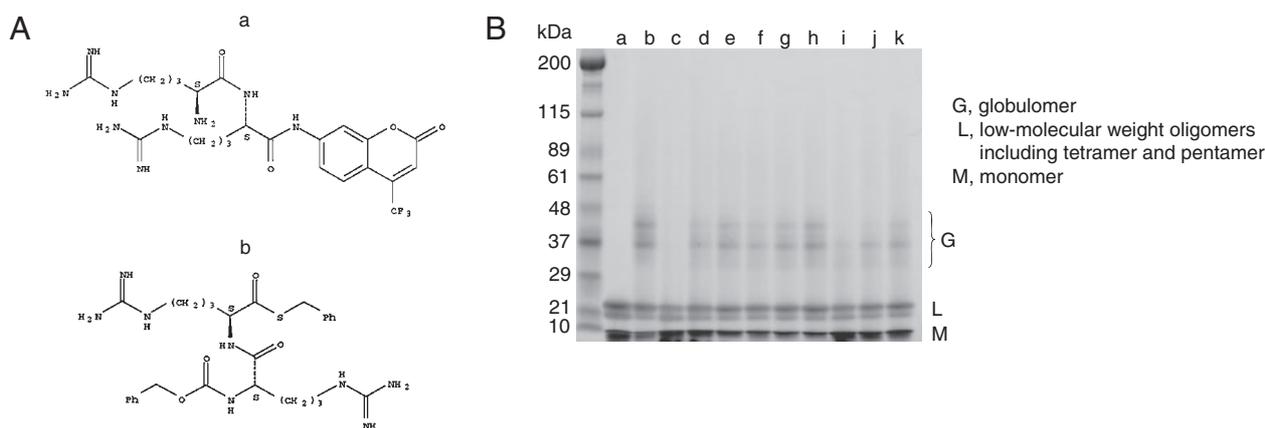
We recently found that arginine-rich short peptides (four or seven residues) bind to soluble 42-residue amyloid  $\beta$  ( $A\beta$ 42) and inhibit globulomer formation using phage display.<sup>7,8)</sup> A tetra-peptide, RFRK, strongly inhibits globulomer formation, and the important factor for inhibitory activity is considered to be arginine residues, as found in previous studies. Although this peptide is expected to be useful in inhibiting toxic globulomer formation, its effect on  $A\beta$  fibril formation is small.<sup>8)</sup> We seek to discover a small molecule that has inhibitory activities against both globulomer and a fibril of  $A\beta$ , because such a molecule is expected to be useful in the development of lead compounds for the reduction of  $A\beta$  toxicity. Most recently, coumarin (2*H*-chromen-2-one), an aromatic molecule found in cinnamon-flavored foods, has been reported to inhibit  $A\beta$  fibril formation.<sup>12)</sup> However, it is not yet known whether coumarin inhibits the globulomer formation of  $A\beta$ 42. Further, a hybrid compound that has both a coumarin moiety and arginine residues within it is expected to exhibit to reduce the aggregated form of  $A\beta$ . Hence we screened hybrid-structured molecules capable of inhibiting  $A\beta$  aggregation from commercially available products. One convenient screening method is to use the SciFinder on-line service.

We found that a chimera-type tri-peptide containing both di-arginine and coumarin bodies, found by the on-line service, inhibited  $A\beta$ 42 aggregation strongly, as expected.

† To whom correspondence should be addressed. Tel: +81-3-5452-6272; Fax: +81-3-5452-6274; E-mail: kamiyo@iis.u-tokyo.ac.jp

\* Present address: IR Free Electron Laser Research Center, Research Institute for Science and Technology (RIST), Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

Abbreviations:  $A\beta$ , amyloid  $\beta$ ; AD, Alzheimer's disease; AMC, 7-amino-4-methylcoumarin; DMSO, dimethyl sulfoxide; HFIP, hexafluoroisopropyl alcohol; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ThT, thioflavine T



**Fig. 1.** Inhibition of A $\beta$ 42 Globulomer by Arginine-Containing Small Compounds.

A, Arginine-containing small molecules selected using SciFinder. a, Arg-Arg-7-amino-4-trifluoromethylcoumarin (RR-AFC). b, Z-Arg-Arg-thiobenzyl ester. Z: benzyloxycarbonyl group. B, SDS-PAGE analysis of the globulomer of A $\beta$ 42 with various compounds. A $\beta$ 42 (0.2 mM) and various compounds (0.2–1.0 mM) were incubated in the presence of 0.1% SDS at 37 °C for 4 h. The solutions were applied on a gradient (5–20%) gel without heat denaturing. a, A $\beta$ 42 only 0 h; b, A $\beta$ 42 only 4 h; c, A $\beta$ 42 with RR-AFC 1.0 mM; d, A $\beta$ 42 with RR-AFC 0.5 mM; e, A $\beta$ 42 with RR-AFC 0.2 mM; f, A $\beta$ 42 with Z-RR-SBzl 1.0 mM; g, A $\beta$ 42 with Z-RR-SBzl 0.5 mM; h, A $\beta$ 42 with Z-RR-SBzl 0.2 mM; i, A $\beta$ 42 with RFRK 1.0 mM; j, A $\beta$ 42 with RFRK 0.5 mM; k, A $\beta$ 42 with RFRK 0.2 mM.

## Materials and Methods

**Materials.** A $\beta$ 42 was purchased from the Peptide Institute (Osaka, Japan). HFIP (hexafluoroisopropyl alcohol), DMSO (dimethyl sulfoxide), SDS (sodium dodecyl sulfate), and ThT (thioflavine T) were from Nacalai Tesque (Kyoto, Japan). Arg-Arg-7-amino-4-trifluoromethylcoumarin and Z-Arg-Arg-thiobenzyl ester were from MP Bio-medicals. (Irvin, CA). 7-Amino-4-methylcoumarin (AMC) was from Nacalai Tesque. Polyacrylamide gel (SuperSep™ Ace 5–20%) were from Wako Pure Chemical Industries (Osaka, Japan). Peptides were from PHJapan (Hiroshima, Japan). The LC-MS data for the synthesized peptides are shown in the Supplemental data (see *Biosci. Biotechnol. Biochem.* Web site).

**Preparation of soluble oligomers of A $\beta$ 42.** A $\beta$ 42 (0.56 mg) was dissolved in 0.27 mL of hexafluoroisopropyl alcohol (HFIP) and dried *in vacuo*.<sup>5)</sup> The dried pellet was redissolved in dimethyl sulfoxide (DMSO) at 1.0 mM as stock solution. To prepare globulomer of A $\beta$ 42, the stock solution was immediately diluted with phosphate-buffered saline (PBS, composed of 137 mM NaCl, 2.7 mM KCl, 12 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.2 mM KH<sub>2</sub>PO<sub>4</sub> at pH 7.4) to 200  $\mu$ M (DMSO, 20%), and this was incubated with 0.1% SDS at 37 °C for 4 h in the presence of peptides or small molecules. To detect the A $\beta$ 42 globulomer, the solutions (7  $\mu$ L) were applied to SDS-PAGE (5–20%) without heat denatures and the gel was stained with Coomassie. Small molecules were dissolved in DMSO at 40 mM as stock solutions and diluted with PBS at appropriate concentrations. Short peptides were dissolved in PBS in 10 mM stock solutions.

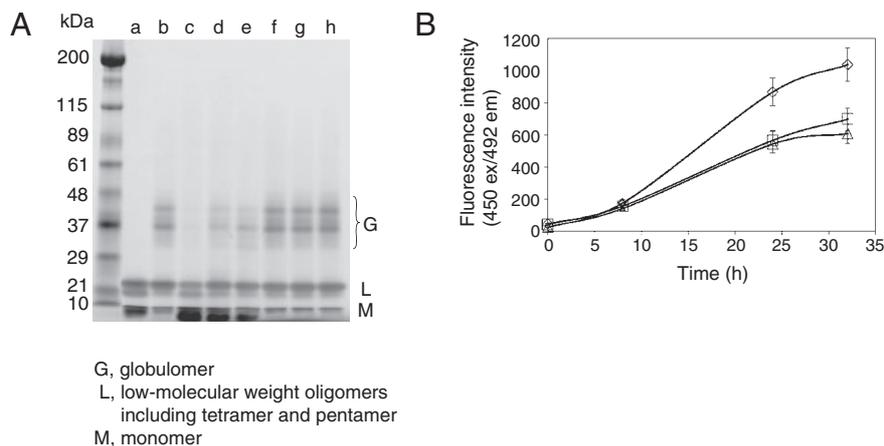
**Preparation of A $\beta$ 42 fibrils and ThT binding assay.** The dried pellets of A $\beta$ 42 after dissolving it in HFIP were redissolved in DMSO at 5.0 mM as stock solution. The solution was diluted with PBS to 0.2 mM (DMSO, 4%) and incubated at 37 °C for 24 h in the presence of the small molecules. The solution was diluted to a final concentration of 8.0  $\mu$ M in PBS and combined with 10  $\mu$ M ThT reagent.<sup>13)</sup> After 10 min of reaction at room temperature, the fluorescence intensity was measured at 492 nm using an excitation wavelength of 450 nm on micro-plate reader MTP800AFC (Corona, Hitachinaka, Japan).

**Search for small molecules using SciFinder.** We logged into the web version (<https://scifinder.cas.org>) of SciFinder, then drew the chemical structure of arginine on the display of substances to be searched, and searched for commercially available compounds containing arginines.

## Results

### *Inhibitory effects of small molecules containing arginines on the aggregation of A $\beta$ 42*

As the upshot of searching for compounds containing arginines, a total of 2,950 molecules were displayed, and several small molecules that had molecular weights of about 500 Da were chosen. Among these, we found a coumarin-containing compound, Arg-Arg-7-amino-4-trifluoromethylcoumarin (RR-AFC, molecular weight 541 Da) (Fig. 1A). Another di-arginine derivative, Z-Arg-Arg-thiobenzyl ester (Z-RR-SBzl, molecular weight 570 Da), was chosen as a reference molecule, because it contained both di-arginine and aromatic groups. RR-AFC contains two arginines and one coumarin moiety. It is used in the assay of neuronal protease activity.<sup>14)</sup> Z-RR-SBzl also contains two arginines, but is protected by aromatic groups at the N- and C-terminals. These compounds were tested for inhibition of globulomer of A $\beta$ 42 by SDS-PAGE (Fig. 1B). As a positive control, RFRK was used.<sup>8)</sup> Globulomer was clearly seen between the 37- and the 48 kDa marker protein after 4 h of incubation of A $\beta$ 42 (0.2 mM) alone (lanes a and b). A monomer (4,514 Da) and a tetramer (18,056 Da), which was seen below the 21-kDa marker protein, were also decreased in amount after incubation. These bands of A $\beta$ 42 were repeatedly detected on the gel.<sup>5,7,8)</sup> On the contrary, globulomer was largely reduced, and instead the monomer and the tetramer remained, in the presence of RR-AFC at a concentration of 1.0 mM (lane c). In the case of a concentration of RR-AFC below 0.5 mM, the globulomer was reduced to approximately half (lanes d and e). These tendencies were almost the same as those for RFRK (lanes i, j, and k). On the other hand, globulomer was seen in the presence of Z-RR-SBzl even at a concentration of 1.0 mM, and it was little inhibited by a concentration of 0.2 mM (lanes f, g, and h). This indicates that Z-RR-SBzl has a weaker effect than RR-AFC on the inhibition of globulomer. Both compounds have arginines and aromatic rings, but the N-terminal of Z-RR-SBzl is blocked by a Z-group and that of RR-AFC is free. In the case of Z-RR-SBzl, a large benzylo-



**Fig. 2.** Effects of Coumarin and RR-AFC on A $\beta$ 42 Aggregation.

A, SDS-PAGE of globulomer formation in the presence of small molecules. A $\beta$ 42 (0.2 mM) and compounds (0.2–1.0 mM) were incubated in the presence of 0.1% SDS at 37 °C for 4 h. The solutions were applied to the gradient gel without heat denaturing. a, A $\beta$ 42 only 0 h; b, A $\beta$ 42 only 4 h; c, A $\beta$ 42 with RR-AFC 1.0 mM; d, A $\beta$ 42 with RR-AFC 0.5 mM; e, A $\beta$ 42 with RR-AFC 0.2 mM; f, A $\beta$ 42 with AMC 1.0 mM; g, A $\beta$ 42 with AMC 0.5 mM; h, A $\beta$ 42 with AMC 0.2 mM. AMC: 7-amino-4-methylcoumarin. B, Time courses of A $\beta$ 42 fibril formation in the presence of small compounds. Solutions (10  $\mu$ L) containing A $\beta$ 42 (0.2 mM) with compounds (0 or 1.0 mM) in PBS (DMSO: 4%) were incubated at 37 °C for 32 h. The resulting solution was mixed with ThT reagent and fluorescent intensities were measured as described under “Materials and Methods.” Assays were performed 3 times, and average values were given. Diamond, A $\beta$ 42 alone; square, A $\beta$ 42 with RR-AFC; triangle, A $\beta$ 42 with AMC.

xy-carbonyl (Z) group at the N-terminal might perturb the interaction of arginine with A $\beta$ 42, and so its binding and inhibitory activities are considered to be somewhat weak. The concentrations of the peptides tested in this study were higher than those of reagents in drug development (on the order of  $\mu$ M). We used sufficiently high concentrations of A $\beta$ 42 to be detected by SDS-PAGE.

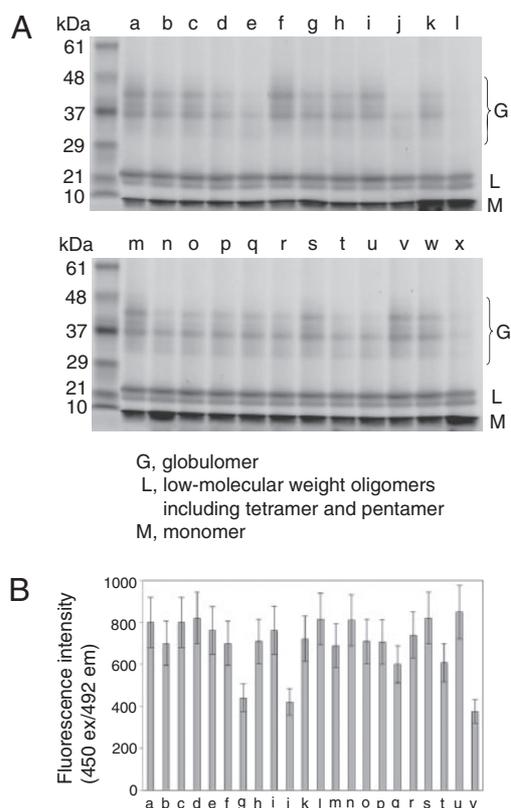
Next, we tested to determine whether coumarin alone inhibits globulomer formation. We purchased 7-amino-4-methylcoumarin (AMC), since 7-amino-4-trifluoromethylcoumarin (AFC) could not be obtained in practical terms. We assumed that the trifluoromethyl group is functionally equal to the methyl group of the coumarin body with regard to molecular size. AMC was dissolved in DMSO, and the solution was diluted with PBS at concentrations of 0.2–1.0 mM, and this was incubated with A $\beta$ 42 (0.2 mM) solutions in the presence of 0.1% SDS. In Fig. 2A, while RR-AFC inhibited globulomer dose-dependently (lanes c, d, and e), AMC did not inhibit it even at a concentration of 1.0 mM (lanes f, g, and h). This indicates that the coumarin moiety does not inhibit the globulomer formation. It can be considered that the R-R moiety of RR-AFC is important in inhibiting the globulomer formation of A $\beta$ 42, as found in previous studies.<sup>7,8</sup> In the presence of RR-AFC at a concentration of 1.0 mM (lane c), both the tetramer and the pentamer (22,570 Da) were also reduced somewhat as compared to the solution of A $\beta$ 42 alone (lane a). This indicates that RR-AFC also inhibits low-molecular weight oligomer formation, including the tetramer and the pentamer of A $\beta$ 42. However, the dimer and the trimer, which have also been reported to be neurotoxic, were not detected by this method, and it is not known whether RR-AFC inhibits the formation of small oligomers.<sup>15,16</sup>

Coumarin derivatives are known to affect fibril formation by A $\beta$ 42.<sup>12</sup> Hence we tested to determine whether RR-AFC and AMC inhibit A $\beta$ 42 fibril formation using ThT reagent (Fig. 2B).<sup>17</sup> A $\beta$ 42 (0.2 mM) was incubated with RR-AFC (1.0 mM) or AMC (1.0 mM) for

32 h at 37 °C. The fluorescence signal from the ThT reagent reached about 1,000 AU in the case of A $\beta$ 42 alone (diamond) at 32 h of incubation. On the contrary, those signals were reduced to about 1/2 in the presence of RR-AFC (square) at 24 h and 32 h of incubations. AMC also reduced the fluorescence signal to the same level (triangle). These results indicate that RR-AFC as well as AMC inhibits the fibril growth of A $\beta$ 42, although those inhibitory effects were weak. However, at 8 h of incubation, A $\beta$  fibril formation was not inhibited by either compound. This indicates that both RR-AFC and AMC have little inhibitory activity against the formation of seeds of A $\beta$  fibrils. RR-AFC inhibited both the globulomer formation and the fibril growth of A $\beta$ 42. On the other hand, AMC affected the fibril only, and hardly inhibited globulomer formation (Fig. 2A). These results indicate that globulomer formation is a different process from fibril formation, and confirm the A $\beta$  aggregation mechanism hypothesized by Barghorn *et al.*<sup>18</sup> They clarified that globulomer formed independently of fibril formation, using Congo red reagent. Both coumarin and Congo red are aromatic compounds that intercalate between  $\beta$ -sheet structures *via* hydrophobic interactions. Consequently, the globulomer is probably formed by hydrophilic driving forces, rather than the hydrophobic interactions that are main causes of fibril formation.

#### *Inhibitory effects of RR-n tri-peptides on the aggregation of A $\beta$ 42*

The molecular size of RR-AFC is almost the same as those of natural RR-n tri-peptides (n indicates any one of 20 amino acids). It is possible that some RR-n tri-peptides have the same inhibitory activities to globulomer as RR-AFC. Hence 20 RR-n tri-peptides were tested for inhibitory activity (Fig. 3A). Some tri-peptides were found to inhibit globulomer formation to about half level of the activity of RR-AFC (lanes l and x). RR-F (lane e), RR-W (lane j), RR-E (lane n), RR-R (lane t), and RR-I (lane u) were weak inhibitors. These



**Fig. 3.** Inhibitory Effects of RR-n Tri-Peptides on the Aggregation of A $\beta$ 42.

**A.** Effects of tri-peptides on the globulomer formation of A $\beta$ 42. A $\beta$ 42 (0.2 mM) and peptides (1.0 mM) were incubated in the presence of 0.1% SDS at 37 °C for 4 h. The solutions were applied on the gradient SDS-PAGE gel without heat denaturing. a, A $\beta$ 42 only; b, A $\beta$ 42 with RRM; c, A $\beta$ 42 with RRL; d, A $\beta$ 42 with RRG; e, A $\beta$ 42 with RRF; f, A $\beta$ 42 with RRK; g, A $\beta$ 42 with RRD; h, A $\beta$ 42 with RRV; i, A $\beta$ 42 with RRA; j, A $\beta$ 42 with RRW; k, A $\beta$ 42 with RRC; l, A $\beta$ 42 with RR-AFC; m, A $\beta$ 42 only; n, A $\beta$ 42 with RRE; o, A $\beta$ 42 with RRN; p, A $\beta$ 42 with RRQ; q, A $\beta$ 42 with RRT; r, A $\beta$ 42 with RRS; s, A $\beta$ 42 with RRP; t, A $\beta$ 42 with RRR; u, A $\beta$ 42 with RRI; v, A $\beta$ 42 with RRY; w, A $\beta$ 42 with RRH; x, A $\beta$ 42 with RR-AFC.

**B.** Effects of tri-peptides on fibril formation by A $\beta$ 42. Solutions (10  $\mu$ L) containing A $\beta$ 42 (0.2 mM) with compounds (0 or 1.0 mM) in PBS (DMSO: 4%) were incubated at 37 °C for 24 h. The resulting solution was mixed with ThT reagent, and the fluorescent intensities were measured as described under "Materials and Methods." Assays were performed 3 times, and average values are given. a, A $\beta$ 42 only; b, A $\beta$ 42 with RRM; c, A $\beta$ 42 with RRL; d, A $\beta$ 42 with RRG; e, A $\beta$ 42 with RRF; f, A $\beta$ 42 with RRK; g, A $\beta$ 42 with RRD; h, A $\beta$ 42 with RRV; i, A $\beta$ 42 with RRA; j, A $\beta$ 42 with RRW; k, A $\beta$ 42 with RRC; l, A $\beta$ 42 with RRE; m, A $\beta$ 42 with RRN; n, A $\beta$ 42 with RRQ; o, A $\beta$ 42 with RRT; p, A $\beta$ 42 with RRS; q, A $\beta$ 42 with RRP; r, A $\beta$ 42 with RRR; s, A $\beta$ 42 with RRI; t, A $\beta$ 42 with RRY; u, A $\beta$ 42 with RRH; v, A $\beta$ 42 with RR-AFC.

results indicate that not only the chromene ring, but also the phenyl ring, indole, and large side chains in tri-peptides have important roles in inhibiting globulomer formation. Finally, the inhibition by tri-peptides of the fibrillation of A $\beta$ 42 was tested using ThT reagents, as described above (Fig. 3B). At the 24 h of incubation of A $\beta$ 42 with tri-peptides, almost all the fluorescent signals of the solutions, except for RR-D (g) and RR-W (j), were at 600–800 AU, almost same levels as that of A $\beta$ 42 alone (a). This indicates that RR-D and RR-W had significant inhibitory activities, as well as RR-AFC (v). As for the inhibitory activity of RR-W, it can be concluded that the tryptophan ring has a role similar to

the chromene ring, because both groups are aromatics. On the other hand, as for the activity of RR-D, it can be concluded that salt bridges between RR-D molecules are formed and affect the association of A $\beta$  molecules, but further examination is necessary to determine the inhibition mechanism.

## Discussion

We succeeded for the first time in identifying an active compound, RR-AFC, that can inhibit both globulomer and fibril aggregations of A $\beta$ 42. In previous studies, phage display was used to screen the binding of molecules to A $\beta$ 42, an active tetra-peptide, RFRK, was found.<sup>7,8)</sup> This tetra-peptide strongly inhibited globulomer formation, but had little effect on A $\beta$  fibril formation. Thus, RR-AFC is better than RFRK for inhibition of the aggregation of A $\beta$ 42. Although phage display is useful in acquiring essential peptide sequences from random libraries, there are several limitations, as for obtaining novel compounds. First, the compounds are limited to structures including 20 amino acids. Secondly, there is a bias in the custom-synthesized random library, due to the synthetic efficiency of DNA (NNK, N = A or U or G or C, K = U or G). On the other hand, chemical libraries, including ones for natural and synthetic products, are available to obtain non-peptide compounds. They are provided by for example the Open Innovation Center for Drug Discovery of The University of Tokyo. SciFinder is considered to be most convenient on-line service for the screening chemical compounds. We decided to use SciFinder by reason of convenience to discover functional compounds.

In a structural study by Yu *et al.* using solution NMR, acidic residues (E22-D23) had important roles in the formation of globulomer of A $\beta$ 42, and the detergent, SDS, induced conformational changes in the A $\beta$  molecule.<sup>9)</sup> Our results indicate that RR-AFC inhibits both globulomer and A $\beta$  fibrils, while coumarin inhibits fibrils only. Based on these results, it can be concluded that the Arg-Arg region is necessary to inhibit globulomer formation *via* ionic interactions, and that another AFC moiety has a role in interrupting the hydrophobic interactions of A $\beta$  molecules *via*  $\pi$ - $\pi$  stacking interactions.<sup>12)</sup> Since SDS is a strong ionic detergent, it can be also concluded that RR-AFC binds specifically to the acidic region including E22-D23 and protects the conformation of A $\beta$  from the detergent.

As found by Glabe *et al.*, small molecules are useful in the characterization of the aggregation of A $\beta$ .<sup>11)</sup> They tested about 40 compounds for inhibition of A $\beta$ 42 aggregation, and proposed two distinct aggregation pathways of A $\beta$ , a soluble oligomer formation pathway and a fibril formation pathway. Our results also indicate that globulomer formation is a process different from fibril formation, as confirmed using the above tri-peptides inhibitors.

In conclusion, we found that a chimera-type tri-peptide, Arg-Arg-coumarin, inhibited both the formation of globulomer and that of fibrils of A $\beta$ 42. It is to be expected that this small compound will prove useful in developing a potent seed molecule for suppression of toxic oligomers of A $\beta$ 42.

## Acknowledgments

We thank Professor Kazuaki Kudo (Institute of Industrial Science, The University of Tokyo) for valuable suggestions. This work was supported in part by Grants-in aid for Challenging Exploratory Research (Grant no. 22651079, to S. K.).

## References

- 1) Selkoe DJ, *Nat. Cell Biol.*, **6**, 1054–1061 (2004).
- 2) Rauk A, *Chem. Soc. Rev.*, **38**, 2698–2715 (2009).
- 3) Chen D, Martin ZS, Soto C, and Schein CH, *Bioorg. Med. Chem.*, **17**, 5189–5197 (2009).
- 4) Sakono M and Zako T, *FEBS J.*, **277**, 1348–1358 (2010).
- 5) Barghorn S, Nimmrich V, Striebinger A, Krantz C, Keller P, Janson B, Bahr M, Schmidt M, Bitner RS, Harlan J, Barlow E, Ebert U, and Hillen H, *J. Neurochem.*, **95**, 834–847 (2005).
- 6) Hillen H, Barghorn S, Striebinger A, Labkovsky B, Müller R, Nimmrich V, Nolte MW, Perez-Cruz C, Auwera I, Leuven F, Gaalen M, Bespalov AY, Schoemaker H, Sullivan JP, and Ebert U, *J. Neurosci.*, **30**, 10369–10379 (2010).
- 7) Kawasaki T, Onodera K, and Kamijo S, *Biosci. Biotechnol. Biochem.*, **74**, 2214–2219 (2010).
- 8) Kawasaki T, Onodera K, and Kamijo S, *Biosci. Biotechnol. Biochem.*, **75**, 1496–1501 (2011).
- 9) Yu L, Edalji R, Harlan JE, Holzman TF, Lopez AP, Labkovsky B, Hillen H, Barghorn S, Ebert U, Richardson PL, Miesbauer L, Solomon L, Bartley D, Walter K, Johnson RW, Hajduk PJ, and Olejniczak ET, *Biochemistry*, **48**, 1870–1877 (2009).
- 10) Ma B and Nussinov R, *J. Biol. Chem.*, **285**, 37102–37110 (2010).
- 11) Necula M, Kayed R, Milton S, and Glabe CG, *J. Biol. Chem.*, **282**, 10311–10324 (2007).
- 12) Soto-Ortega DD, Murphy BP, Gonzalez-Velasquez FJ, Wilson KA, Xie F, Wang Q, and Moss MA, *Bioorg. Med. Chem.*, **19**, 2596–2602 (2011).
- 13) Yoshihara T, Takiguchi S, Kyuno A, Tanaka K, Kuba S, Hashiguchi S, Ito Y, Hashimoto T, Iwatsubo T, Tsuyama S, Nakashima T, and Sugimura K, *J. Biochem.*, **143**, 475–486 (2008).
- 14) Kato T, Yajima R, Sato N, Takahashi K, Shimizu C, and Chikuma T, *Neurochem. Int.*, **32**, 163–170 (1998).
- 15) Jin M, Shepardson N, Yang T, Chen G, Walsh D, and Selkoe DJ, *Proc. Natl. Acad. Sci. USA*, **108**, 5819–5824 (2011).
- 16) Nogalska A, D'Agostino C, Engel WK, Klein WL, and Askanas V, *Acta Neuropathol.*, **120**, 661–666 (2010).
- 17) Bourhim M, Kruzel M, Srikrishnan T, and Nicotera T, *J. Neurosci. Methods*, **160**, 264–268 (2007).
- 18) Gellermann GP, Byrnes H, Striebinger A, Ullrich K, Mueller R, Hillen H, and Barghorn S, *Neurobiol. Dis.*, **30**, 212–220 (2008).