Electronic Supplementary Information (ESI)

Role of the carboxy groups of triterpenoids in their inhibition of the nucleation of amyloid β42 required for forming toxic oligomers

Kazuma Murakami,[a] Takuya Yoshioka,[a] Shiori Horii,[a] Mizuho Hanaki,[a] Satohiro Midorikawa,[b] Shinji Taniwaki,[b] Hiroki Gunji,[b] Ken-ichi Akagi,[c] Taiji Kawase,[d] Kenji Hirose[d] and Kazuhiro Irie*[a]

[a]Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[b]Alps-Pharmaceutical Industry Co., Ltd., Gifu 509-4241, Japan
[c]National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan
[d]Nihon Waters, K. K., Tokyo 140-0001, Japan

*Corresponding author
Kazuhiro Irie, Ph.D., Tel: +81-75-753-6281. Fax: +81-75-753-6284.
E-mail: irie@kais.kyoto-u.ac.jp.

[Contents]

Supplementary discussion

Regarding the atomic resolution based on the NMR results (Fig. 2a,b), the C-27 ferulate moiety of **1** was deduced to interact with these β-sheet region including benzene rings (Gln15-Ala21) of Aβ42 due to its inherent hydrophobicity and planarity derived from α,β-unsaturated carbonyl groups *via* π-π stacking, similarly to the case of curcumin.[1] Instead, the basic amino acid residues (Arg5 or Lys16) could also be assumed to contribute to the formation of salt bridge with C-28 carboxy group of **1**.

Given IM-MS data of Aβ42 using **1**~**3** (Fig. 2c-h), The remarkable perturbation of chemical shifts in the neighboring residues to Lys16, but not Lys28, observed in SOFAST-HMQC studies (Fig. 2a,b), suggest a coordinated structural changes of intermolecular β-sheet regions, induced from the salt bridge formation with Lys28 residue. Alternatively, adduct formation is more likely to occur at Lys16 than that at Lys28. Our group have reported that the toxic oligomers could originate from toxic conformer possessing the turn structure at positions 22 and 23,[2] which might be one of the targets by **1**.

Experimental procedures

1. Thioflavin-T (Th-T) fluorescence assay

The aggregative ability of each amyloid β42 (Aβ42) was evaluated by the thioflavin-T (Th-T; Sigma-Aldrich, St. Louis, MO, USA) fluorescence assay developed by Naiki and Gejyo.[3] The basic procedure has been described elsewhere[4], with the exception of the description of continuous-type measurement. For example, 435 μL of phosphate buffered saline (PBS: 50 mM sodium phosphate and 100 mM NaCl, pH 7.4) was aliquoted into a 1.5 mL tube, followed by the addition of 10 μL of 1 mM Th-T solution in distilled water and 5 μL of each test sample [**1**: uncarinic acid C,[5] **2**: C-27 methyl alcohol of **1**,[5] **3**: C-28 methyl alcohol of **1**,[5] **4**: asiatic acid (Tokyo Kasei, Tokyo, Japan), **5**: α-amyrin (Extrasynthese, Lyon, France), **6**: rhein (Tokyo Kasei), and **7**: chrysophanic acid (Tokyo Kasei)] (2 mM), dissolved with ethanol (**1**–**5**) or DMSO (**6**, **7**). Then, the 50 μL solution of Aβ42 (100 μM in 0.1% NH_4OH), synthesized as reported previously,[4] was added to the tube, so that the

final concentration was 10 μM of Aβ42, 20 μM of each sample, and 20 μM of Th-T. After pre-incubation on ice for 15 min, the solution was aliquoted into a 96-well black plate (Thermo Scientific, Rockford, IL, USA) before sequentially measuring the fluorescence at 430 nm excitation and 485 nm emission (100 μL/well) at 23 °C with agitation at 10-min intervals using a microplate reader (Fluoroskan Ascent; Thermo Scientific). Th-T relative fluorescence was expressed after subtraction of vehicle control without Aβ42.

For treatment with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), Aβ42 was first dissolved in HFIP at 1 mg/mL before incubating at room temperature for 30 min. The resultant solution was sonicated for 5 min, and then dispensed in desired volumes. The solution was left at room temperature overnight to volatilize the HFIP. This was followed by centrifugal concentration to obtain HFIP-treated Aβ42 (HFIP-Aβ42), which was monomeric and its purity was >98% (Fig. S1a). The nucleation time (*ca*. 2 h) of non-treated Aβ42 was earlier than that (*ca*. 4 h) of HFIP-Aβ42 (Fig. S1b), and these results are generally in agreement with our previous study.[6]

2. Circular dichroism (CD) spectrometry

CD spectra were measured using a 0.1 mm quartz cell as described elsewhere.[4] HFIP-Aβ42 solution in 0.1% NH_4OH at 250 μM and **1** solution in ethanol at 5 mM were diluted with PBS to a final concentration of 25 and 50 μM, respectively, and were then incubated without or with **1** in PBS at 37 ℃. After several intervals, an aliquot (200 μL) was loaded into the quartz cell, and the CD spectrum was recorded at 190–250 nm. The spectra of Aβ42 are shown after subtraction of the spectrum of the vehicle alone, and those in the presence of **1** are shown after subtraction of the spectrum for **1** alone.

3. MTT assay

SH-SY5Y cells (ATCC, Manassas, VA, USA), maintained in a mixed medium containing equal amounts of Eagle's minimal essential medium (EMEM; Wako) and Ham's F12 medium (Wako) containing 10% fetal bovine serum, were used as one of the

typical neuronal cell models to estimate the neurotoxicity of Aβ42 with slight modifications to the described method.[7] In brief, HFIP-Aβ42 and **1** were dissolved in 0.1% NH_4OH or in ethanol to make a 12× stock before being diluted with culture medium to the desired final concentration (1% ethanol). After pre-incubating 120 μL of Aβ42 with **1** for 30 min at room temperature, the culture medium used on near-confluent cells (1×10^4 cells/well) for overnight adaptation was exchanged with the pre-incubated solution (120 μL). After incubation for 24 h at 37 °C, 15 μL/well of Dye solution in CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) was added to the cells, followed by incubation for 4 h at 37 °C. The solubilization/stop solution (100 μL/well) was subsequently added to the cells. The cell lysate was subsequently incubated overnight in the dark at room temperature before performing measurements at 570 nm with a microplate reader (Multiskan FC; Thermo Scientific). The absorbance obtained by adding the vehicle (0.1% NH_4OH + 1% ethanol) was taken as 100%.

4. ^{1}H-^{15}N SOFAST-HMQC NMR measurement

^{1}H-^{15}N SOFAST-HMQC measurement was carried out mainly according to our previous report.[6] Uniformly ^{15}N-labeled Aβ42 (67.7 μg; rPeptide, Bogart, GA, USA) treated with HFIP was dissolved at 250 μM in 60 μL of 10 mM NaOH, containing 10 mM EDTA. **1** was dissolved in ethanol at 1 mM, and 30 μL of the solution was diluted with 504 μL of 5 mM phosphate buffer (PB: 5 mM sodium phosphate, pH 6.98) containing 1% D_2O (6 μL). Then, 250 μM of the Aβ42 solution (60 μL) was added, so that the final concentrations of Aβ42 and **1** were 25 μM and 50 μM, respectively. Peaks were assigned by referring to previous findings.[6,8-10] Each distance in the ^{1}H-^{15}N chemical shifts between the cross peaks of Aβ42 alone and Aβ42 in the presence of **1** was calculated by the Pythagorean theorem. ^{15}N chemical shifts were scaled one-tenth relative to ^{1}H chemical shifts because the measurement range (6.5–8.6 ppm) of observations of ^{1}H was approximately one-tenth of that (107–128 ppm) of ^{15}N.

5. Ion mobility–mass spectrometry (IM-MS).

Aβ42 or Lys16Nle,Lys28Nle-Aβ42[4] was dissolved in 0.1% NH_4OH at 400 μM and each test sample was solubilized in ethanol at 8 mM, followed by a 10-fold and 100-fold dilution with 25 mM ammonium acetate (pH 7.4), respectively. The resultant solution (Aβ42: 40 μM, test sample: 80 μM) was centrifuged for 4 min at 2,000 *g* (4 °C) before infusion into the MS apparatus using a glass capillary (Nanoflow Probe Tip, Waters). Mass spectra and ion mobility experiments were accomplished on SYNAPT G2-Si HDMS (Waters) using a nanoelectrospray as an ionization source. The instrument was operated in negative ion mode with a capillary voltage of 1.0 kV, a sample cone voltage of 10 V, a source temperature of 50 °C, and a desolvation temperature of 50 °C. For the ion mobility measurement, nitrogen gas was used in the ion mobility cell, and the cell pressure was maintained at approximately 2.95 mbar with a wave velocity of 300–1,000 m/s and a wave height of 10–40 V. Data acquisition and processing were performed with the MassLynx (V4.1) and DriftScope (V2.8) software supplied with the instrument. The CsI cluster ions were used for m/z scale as a calibrator.

Supplementary references

1 Y. Masuda, M. Fukuchi, T. Yatagawa, M. Tada, K. Takeda, K. Irie, K. Akagi, Y. Monobe, T. Imazawa and K. Takegoshi, *Biorg. Med. Chem.*, 2011, **19**, 5967-5974.

2 Y. Masuda, S. Uemura, R. Ohashi, A. Nakanishi, K. Takegoshi, T. Shimizu, T. Shirasawa and K. Irie, *ChemBioChem*, 2009, **10**, 287-295.

3 H. Naiki and F. Gejyo, *Methods Enzymol.*, 1999, **309**, 305-318.

4 M. Sato, K. Murakami, M. Uno, Y. Nakagawa, S. Katayama, K. Akagi, Y. Masuda, K. Takegoshi and K. Irie, *J. Biol. Chem.*, 2013, **288**, 23212-23224.

5 T. Yoshioka, K. Murakami, K. Ido, M. Hanaki, K. Yamaguchi, S. Midorikawa, S. Taniwaki, H. Gunji and K. Irie, *J. Nat. Prod.*, 2016, **79**, 2521-2529.

6 M. Hanaki, K. Murakami, K. Akagi and K. Irie, *Bioorg. Med. Chem.*, 2016, **24**, 304-313.

7 K. Murakami, M. Tokuda, T. Suzuki, Y. Irie, M. Hanaki, N. Izuo, Y. Monobe, K. Akagi, R. Ishii, H. Tatebe, T. Tokuda, M. Maeda, T. Kume, T. Shimizu and K. Irie, *Sci. Rep.*, 2016, **6**, 29038.

8 L. Hou, H. Shao, Y. Zhang, H. Li, N. K. Menon, E. B. Neuhaus, J. M. Brewer, I. J. Byeon, D. G. Ray, M. P. Vitek, T. Iwashita, R. A. Makula, A. B. Przybyla and M. G. Zagorski, *J. Am. Chem. Soc.*, 2004, **126**, 1992-2005.

9 R. P. Friedland, J. M. Tedesco, A. C. Wilson, C. S. Atwood, M. A. Smith, G. Perry and M. G. Zagorski, *J. Biol. Chem.*, 2008, **283**, 22550-22556.

10 D. K. Weber, M. A. Sani and J. D. Gehman, *Amino Acids*, 2014, **46**, 2415-2426.

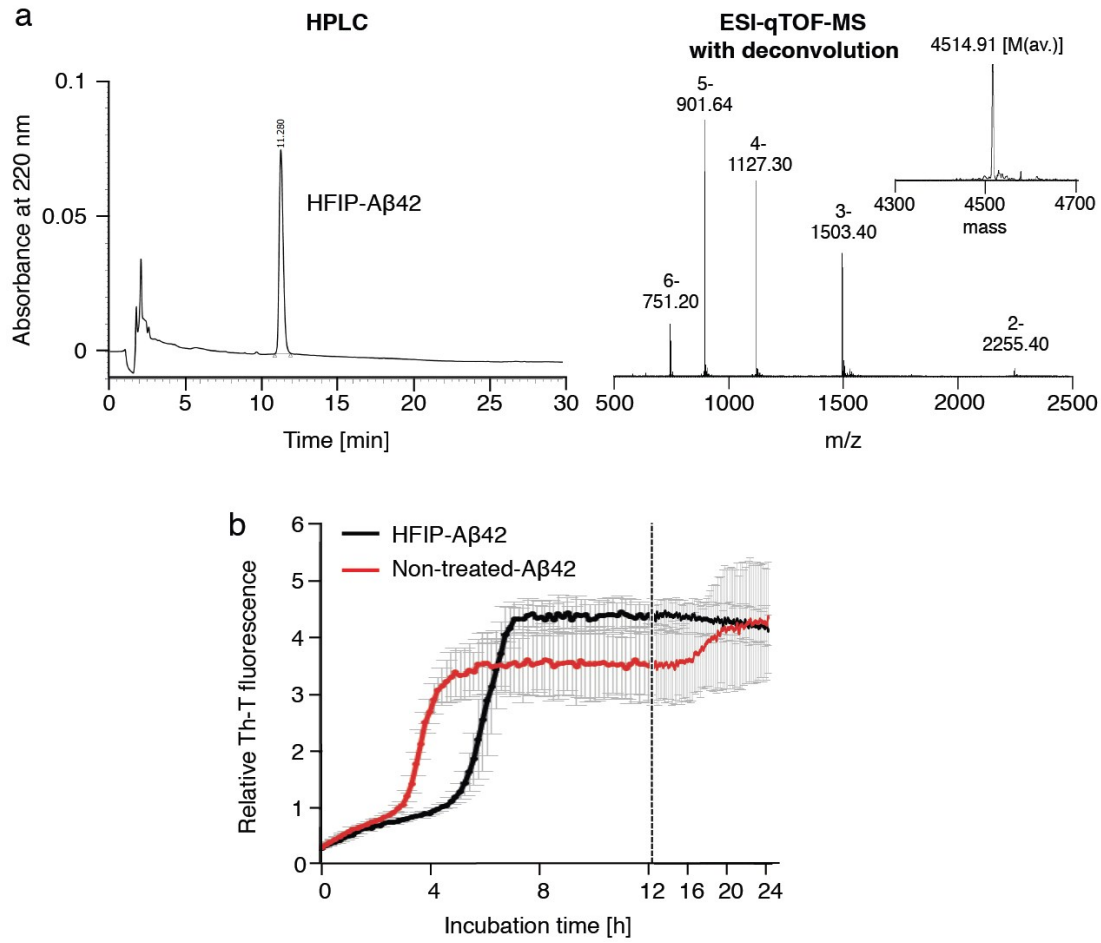

Fig. S1 (a) HPLC profile and ESI-qTOF-MS data with deconvolution of HFIP-treated Aβ42, synthesized in our group.[4] HPLC condition: Develosil ODS-UG-5 (100 x 6 mm I.D.), 1 mL/min, UV 220 nm, 10-50% acetonitrile containing 0.1% NH_4OH (30 min linear gradient), 625 pmole/25 μL (0.1% NH_4OH) injection. HFIP-treated Aβ42, m/z (calcd for av. mass, 4514.08). (b) Sequential Th-T aggregation test with a 10-min interval. HFIP-treated Aβ42 or non-treated Aβ42 (10 μM) was incubated in the presence of Th-T (20 μM) at room temperature (23 °C). Data are presented as the mean ± SD (n = 4).

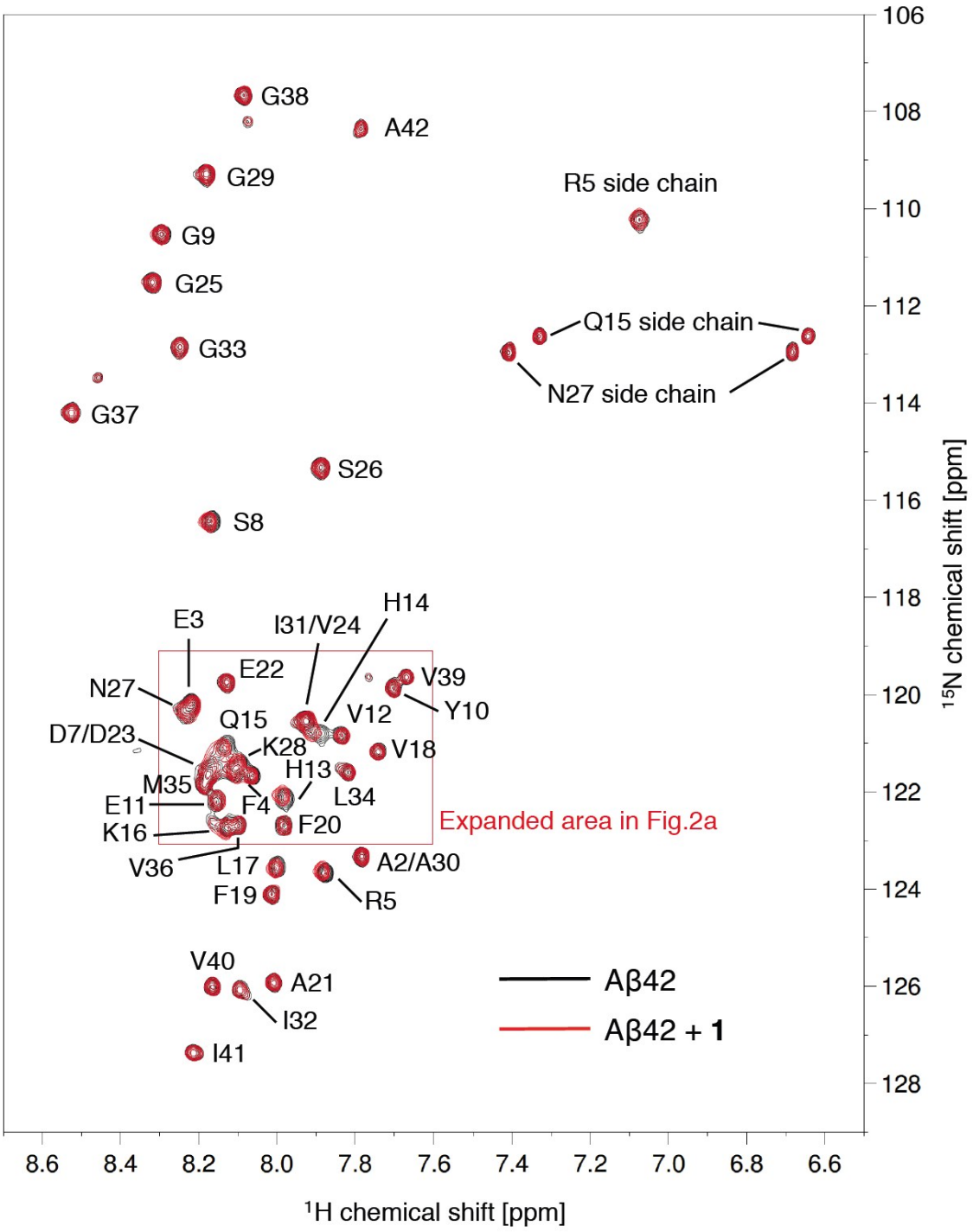

Fig. S2 Full spectra of ^{1}H-^{15}N SOFAST-HMQC NMR of Aβ42 in the absence or presence of **1**, the expanded version of which is shown in Fig. 2a in the main text. Black cross peaks, Aβ42 alone; red cross peaks, Aβ42 treated with **1**.

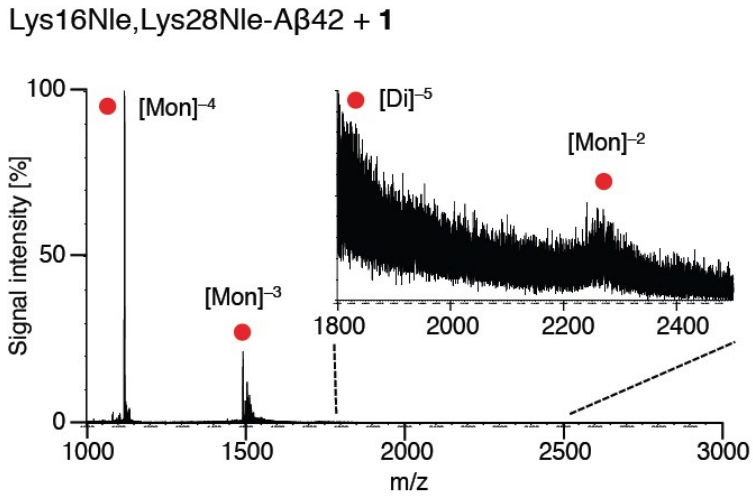

Fig. S3 NanoESI-TOF-MS of Lys16Nle,Lys28Nle-Aβ42 (40 μM) with **1** (80 μM). Peaks for Aβ42 are noted with red circles. Nle = norleucine