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Crocetin inhibits beta-amyloid fibrillization and stabilizes beta-amyloid oligomers

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ABSTRACT

Aggregation of a peptide, beta-amyloid (Aβ), is a hallmark molecular process found in Alzheimer's disease (AD). During Aβ aggregation, oligomeric and fibrillar Aβ are formed, and these molecular self-assembly steps are implicated in generation of toxic effects in AD. Crocetin is a natural carotenoid dicarboxyl acid displaying various pharmaceutical effects and may be co-localized with Aβ mediated by human serum albumin. In the study presented here, we examined the effects of crocetin on Aβ aggregation in three different molecular pathways. Our results demonstrate that crocetin inhibited Aβ fibril formation and destabilized pre-formed Aβ fibrils. Moreover, crocetin caused stabilization of Aβ oligomers and prevented their conversion into Aβ fibrils. Our study reveals potential pathological and pharmaceutical implication of crocetin in AD and suggests possible application of crocetin for currently limited structural studies on unstable Aβ oligomers.

1. Introduction

Aggregation of a 4 kDa peptide, β-amyloid (Aβ, Fig. 1A), is believed to be implicated in the pathology of Alzheimer's disease (AD) [1,2]. During aggregation, natively unfolded Aβ monomers self-assemble to form soluble oligomeric intermediates, which then further aggregate to amyloid fibrils [1,2]. An increasing body of studies demonstrates that Aβ oligomers rather than Aβ monomers or Aβ fibrils are major toxic agents in AD [3–5]. Crocetin (8,8′-diapocarotenedioic acid) is a natural carotenoid dicarboxyl acid found in the crocus flower. Crocetin contains a conjugated carbon chain and two carboxyl groups at its ends (Fig. 1B). Numerous studies revealed beneficial pharmaceutical effects of crocetin, such as enhancement of oxygen transport [6], inhibition of tumor cell proliferation [7] and reduction of severity of the atherosclerosis [8]. Crocetin can also provide neuroprotective effects by reducing the production of various neurotoxic molecules from activated microglia [9]. When orally administered, crocetin can be rapidly absorbed into the blood plasma [10] and may bind to human serum albumin (HSA) [7], the principal extracellular protein in blood plasma [11]. HSA is also an important regulator of Aβ flux between the brain and the periphery: HSA may shift the equilibrium of Aβ across the blood–brain barrier (BBB) [12] by preferentially binding to Aβ oligomers [13]. As such, there is a high likelihood that crocetin and Aβ coexist in a local space mediated by HSA. Unfortunately, no study of the effects of crocetin on Aβ aggregation has yet to be carried out.

In the study presented here, we examined Aβ aggregation in the presence and absence of crocetin in three different aggregation pathways. Our results demonstrate that crocetin inhibited Aβ fibril formation and destabilized pre-formed Aβ fibrils. Moreover, crocetin caused stabilization of pre-formed Aβ oligomers and prevented their conversion to Aβ fibrils. Pathophysiological and pharmaceutical relevance of crocetin in AD and potential application of crocetin for currently limited structural studies on unstable Aβ oligomers were also discussed.

2. Materials and methods

2.1. Materials

Lyophilized Aβ containing 40 residues, D1-V40, was purchased from W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. PG46 was synthesized by solid-phase chemistry, purified by reverse-phase HPLC, lyophilized and confirmed by MALDI-TOF mass spectrometry, as described previously [14]. An oligomer conformation-specific polyclonal antibody, A11, was purchased from Invitrogen. Crocetin (8,8′-diapocarotenedioic acid) was purchased from MP Biomedicals, LLC. All other chemicals were purchased from Sigma–Aldrich unless otherwise stated.

Abbreviations: Aβ, beta-amyloid; AD, Alzheimer's disease; HSA, human serum albumin; BBB, blood–brain barrier; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HFIP, hexafluoroisopropanol; PBSA, phosphate-buffered saline with azide; DMSO, dimethyl sulfoxide; ThT, thioflavin T; TEM, transmission electron microscopy; CD, circular dichroism; SDS, sodium dodecyl sulfate; TCEP, tris(2-carboxyethyl)phosphine; EDT, 1,2-ethanedithiol.

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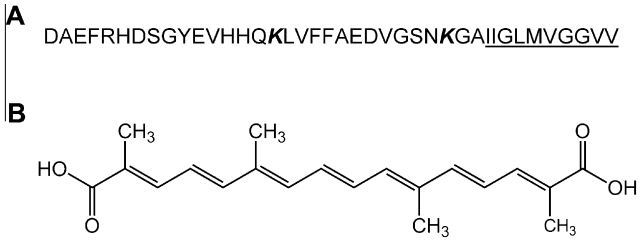

Fig. 1. (A) The amino acid sequence of Aβ containing 40 residues and (B) the chemical structure of crocetin. In (A), Lys 16 and Lys 28 are shown in bold italic and the Aβ C-terminal domain (i.e., residues 31–40) is underlined.

2.2. Aβ sample preparation and incubation

Aβ samples were prepared according to established protocols [15] where Aβ powders were pre-treated with hexafluoroisopropanol (HFIP) and then re-lyophilized. For freshly-prepared Aβ samples, the HFIP-treated, re-lyophilized Aβ was re-solubilized with 10 mM NaOH at 1.75 mg/ml (=406 μM) for 10 min. Aβ solution in NaOH was then 7-fold diluted into phosphate-buffered saline with azide (PBSA, 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, 0.02% (w/v) NaN_3, pH 7.4). To prepare pre-formed Aβ oligomeric samples, the HFIP-treated, re-lyophilized Aβ was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml (=4.65 mM) for 30 min, followed by 20-fold dilution into PBSA. Aβ solutions were incubated at 37 °C for ~3 days without shaking and supernatants then collected after centrifugation. Pre-formed Aβ fibril samples were obtained by ~2-month incubation without shaking at 25 °C of fresh Aβ samples at 58 μM, which were prepared as described above. Samples were then centrifuged for 20 min to discard soluble fractions. Insoluble pellets were subsequently washed multiple times with PBSA and resuspended with the same buffer. In all cases, PBSA was pre-filtered through 0.22-μm filters and samples were prepared in glass vials or siliconized tubes. Aβ samples prepared as described above were diluted, if necessary, at the designated concentrations and mixed with concentrated crocetin solutions at a final ratio of 1:0.33, 1:1 and 1:3. Samples were then incubated at the indicated temperatures without shaking.

2.3. Thioflavin T (ThT) fluorescence and other sample characterizations

Twenty microliters of sample (i.e., Aβ solutions with or without crocetin) was mixed with 10 μl of 0.1 mM ThT solution in water and 170 μl of PBSA per 200 μl of the final volume. ThT fluorescence of samples was then immediately measured using a Photon Technology QuantaMaster QM-4 spectrofluorometer. Excitation wavelength was 440 nm and emission was monitored at 485 nm.

Measurements of FlAsH fluorescence using PG46, dot blot assays using A11, transmission electron microscopy (TEM) and circular dichroism (CD) spectroscopy were performed as described previously (also see Supplementary material for details of FlAsH fluorescence measurements) [14].

3. Results

3.1. Effects of crocetin on fresh Aβ

We first characterized aggregation of freshly-prepared Aβ at 58 μM during incubation at 37 °C using fluorescence of thioflavin T (ThT), a fluorescent dye specific for amyloid fibrillar structures [16]. As expected, samples containing freshly-prepared Aβ displayed low ThT fluorescence at the beginning of incubation (Fig. 2A), indicating the lack of significant amyloid fibrils in these samples. The lack of any large aggregates in these samples was also confirmed by transmission electron microscopy (TEM) (Fig. 3A left). Upon further incubation at 37 °C, Aβ in these samples underwent significant aggregation to form amyloid fibrils as reflected by a ThT fluorescence increase (Fig. 2A). The presence of mature fibrils in Aβ samples after 21 days of incubation was verified using TEM (Fig. 3A middle). Aggregation of freshly-prepared Aβ was also examined in the presence of crocetin at three molar ratios (1:0.33, 1:1 and 1:3) under the otherwise same incubation condition. Interestingly, crocetin significantly inhibited Aβ fibril formation as determined by ThT fluorescence, particularly at the late stage of aggregation, at all three concentration ratios we tested (Fig. 2A). The reduced ThT fluorescence of Aβ samples caused by the presence of crocetin was not due to competition between ThT and crocetin for binding to Aβ fibrillar structures: no significant increase in ThT fluorescence was observed from samples containing Aβ fibrils and crocetin, when a higher concentration of ThT was added in the assay buffer (data not shown). We also examined whether the observed reduction of ThT fluorescence by crocetin could predominantly be due to inner filter effects resulting from absorbance of crocetin at λ_{ex} (=440 nm) and λ_{em} (=485 nm) used for ThT fluorescence measurements. According to previous literatures [17–20] and the measured crocetin absorbance at 440 and 485 nm, the presence of 174 μM (=3 × 58 μM) of crocetin in Aβ samples was estimated to cause additional reduction of ThT fluorescence by ~25% due to inner filter effects under our ThT fluorescence assay conditions (see "*Inner filter effects*" in Supplementary

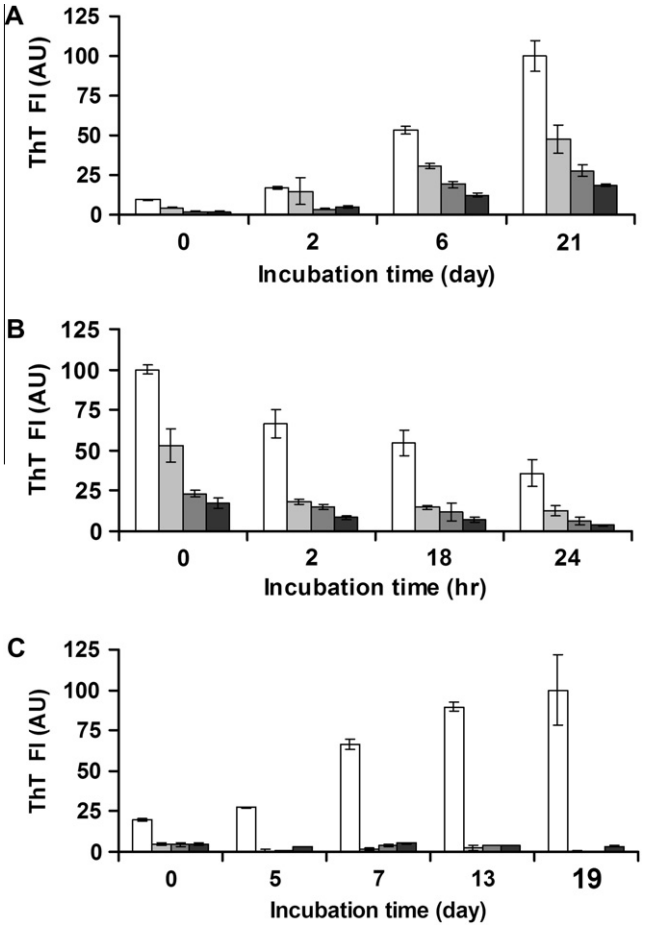

Fig. 2. Thioflavin T (ThT) fluorescence of samples of Aβ only (white block) and a mixture of Aβ + crocetin at a ratio of 1:0.33 (light gray block), 1:1 (dark gray block) and 1:3 (black block) during incubation. In (A), freshly-prepared Aβ at 58 μM in aqueous buffer was incubated in the presence and absence of crocetin at 37 °C. In (B) pre-formed Aβ fibrils or (C) pre-formed Aβ oligomers at 23 μM in aqueous buffer were incubated in the presence and absence of crocetin at 25 °C. The error bars represent one standard deviation of triplicates.

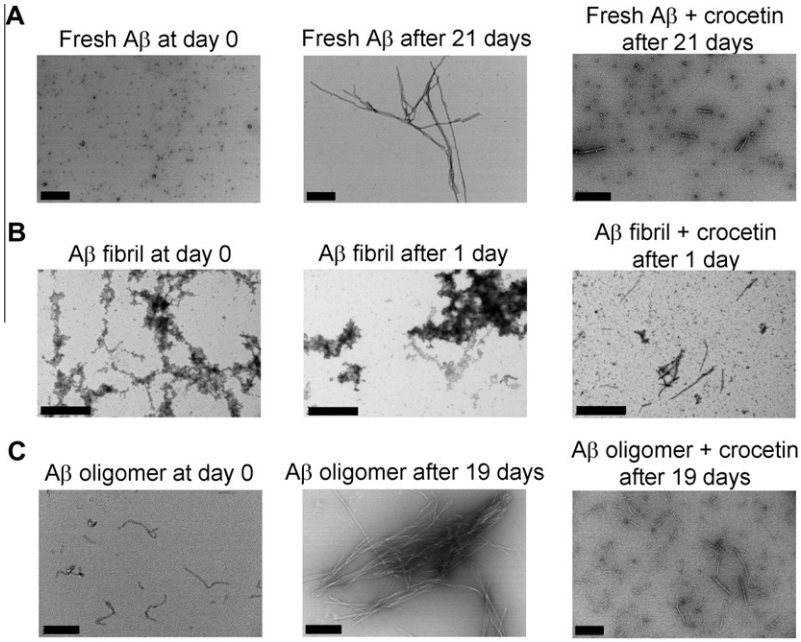

Fig. 3. Representative TEM images of samples containing (A) freshly-prepared Aβ at day 0 (left), freshly-prepared Aβ after 21 days of incubation at 37 °C in the absence (middle) and presence (right) of crocetin at the 1:3 ratio; (B) pre-formed Aβ fibrils at day 0 (left), pre-formed Aβ fibrils after 1 day of incubation at 25 °C in the absence (middle) and presence (right) of crocetin at the 1:3 ratio; (C) pre-formed Aβ oligomers at day 0 (left), pre-formed Aβ oligomers after 19 days of incubation at 25 °C in the absence (middle) and presence (right) of crocetin at the 1:3 ratio. Aβ concentrations in samples during incubation were (A) 58 μM or (B and C) 23 μM. Scale bars represent (A and C) 200 nm or (B) 1000 nm.

material for details). In contrast, only $\leqslant\sim$10% additional reduction of ThT fluorescence should occur resulting from inner filter effects when $\leqslant\sim$70 μM of crocetin is present in Aβ samples. No other spectral interference of crocetin with ThT fluorescence was detected (data not shown). Taken together, the observed reduction (i.e., $\geqslant\sim$50%) of ThT fluorescence of Aβ samples by crocetin was much larger than inner filter effects at all three crocetin concentrations we tested after 6 and 21 days of incubation (Fig. 2A), indicating that inhibition of Aβ fibril formation by crocetin is significant. The Aβ fibrillization-inhibitory effect of crocetin was further confirmed by TEM (Fig. 3A right). Prevention of Aβ fibril formation by crocetin was also detected when freshly-prepared Aβ at 23 μM was incubated at 25 °C with crocetin at the aforementioned three ratios (1:0.33, 1:1 and 1:3), as monitored by ThT fluorescence (Fig. S1). As described above, inner filter effects of crocetin under this condition are minimal, representing only $\leqslant$10% additional reduction of ThT fluorescence.

3.2. Effects of crocetin on pre-formed Aβ fibrils

We then evaluated effects of crocetin on Aβ fibrils pre-formed in the absence of crocetin. When incubated at 23 μM and 25 °C, pre-formed Aβ fibrils in solution showed a modest decrease in ThT fluorescence over time (Fig. 2B), presumably due to partial dissociation, with slight, if any, changes in fibrillar networks (Fig. 3B left and middle, and Fig. S2 left and middle). When crocetin was present in pre-formed Aβ fibril samples, a larger extent of destabilization of Aβ fibrils was detected immediately (Fig. 2B). Moreover, Aβ fibrillar networks were significantly disrupted by crocetin (Fig. 3B right and Fig. S2 right). Taken together, our results suggest that crocetin destabilized pre-formed Aβ fibrils and disrupted Aβ fibril networks.

3.3. Effects of crocetin on pre-formed Aβ oligomers

Next, we examined effects of crocetin on Aβ oligomers pre-formed in the absence of crocetin. A predominant fraction ($\sim$80%) of these pre-formed Aβ samples was oligomeric (>50 kDa) displaying protofibrillar morphologies (Fig. 3C left) with the remainder comprising Aβ monomers [14]. When no crocetin was present in samples, pre-formed Aβ oligomers at 23 μM were converted to mature Aβ fibrils after 19 days of incubation at 25 °C, as determined by ThT fluorescence (Fig. 2C) and TEM (Fig. 3C middle). In contrast, crocetin significantly inhibited conversion of these pre-formed Aβ oligomers into Aβ fibrils (Figs. 2C and 3C right).

We then characterized the effects of crocetin on conformational states of Aβ oligomers through dot blot assays using an antibody A11. Note that A11 recognizes specific conformational structures of Aβ oligomers but not those of Aβ monomers or Aβ fibrils [3]. As reported previously [14], the pre-formed Aβ oligomers prepared as described in Section 2 were initially A11-positive (Fig. 4A for Aβ only at day 0), but then became A11-negative as these oligomers were converted into Aβ fibrils during incubation (Fig. 4A for Aβ only at days 13 and 19). In contrast, A11 responses of Aβ oligomers became stronger immediately upon addition of crocetin and then remained significantly unchanged even after 19 days of incubation in the presence of an equimolar or 3-fold excess concentration of crocetin (Fig. 4A for Aβ + crocetin). The implication is that A11-positive conformational epitopes present in Aβ oligomers were significantly preserved in the presence of $\geqslant$equimolar concentrations of crocetin. We further sought to verify A11-dot blot results using another independent method. To this end, we probed the oligomeric state of Aβ samples after 13 days of incubation with or without crocetin using an Aβ oligomer-specific fluorescent peptide probe, PG46, that our lab previously developed [14]. We chose samples incubated for 13 days rather than 19 days for probing by PG46 since larger fibrils produced from longer incubation may cause a significant light scattering effect, which complicates interpretation of results, during fluorescence measurements. PG46 displayed an $\sim$1.6 to 2-fold increase in fluorescence of a biarsenical dye, FlAsH, upon binding to A11-positive Aβ oligomers at $\geqslant$23 μM [14], whereas no such increase was observed when PG46 was mixed with Aβ monomers or Aβ fibrils at similar concentration ranges [14]. Consistent with the previous observation, PG46 displayed a

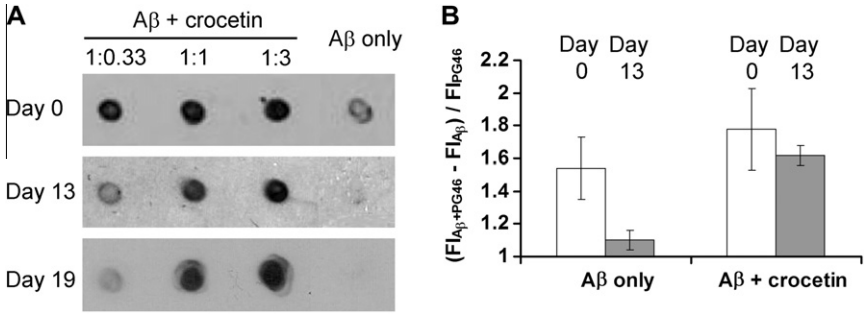

Fig. 4. (A) Dot blot assays using A11 of pre-formed Aβ oligomers only and a mixture of pre-formed Aβ oligomers + crocetin at a ratio of 1:0.33, 1:1 and 1:3. Aβ concentrations in samples were 23 μM. Samples were incubated at 25 °C and blotted on membranes after indicated incubation times. (B) FlAsH fluorescence of PG46 when mixed with pre-formed Aβ oligomers only (Aβ only) and a mixture of pre-formed Aβ oligomers + crocetin at a 1:3 ratio (Aβ + crocetin). Samples containing Aβ at 23 μM with or without crocetin were incubated at 25 °C for 13 days. These samples were then mixed with PG46 for FlAsH measurements. FlAsH fluorescence was measured with the excitation wavelength of 508 nm and the fluorescence intensity was measured at 536 nm. The data obtained were plotted for the ratio of (FlAsH fluorescence intensity of a mixture of Aβ plus PG46–FlAsH fluorescence intensity of Aβ only)/(FlAsH fluorescence intensity of PG46 only). As such, this ratio must be 1 for PG46 only samples. The errors were evaluated by the propagation of error method.

~1.6-fold FlAsH fluorescence increase when mixed with pre-formed Aβ oligomers at the beginning of incubation (Fig. 4B), while exhibiting no significant increase with those incubated for 13 days during which most Aβ oligomers aggregated to Aβ fibrils as shown in Figs. 2C, 4A and B. In contrast, PG46 displayed a ~1.6–1.8-fold FlAsH fluorescence increase with pre-formed Aβ oligomers not only at the beginning of incubation but also after 13 days when crocetin was present in samples at the 3-fold excess concentration. These results further support the notion that the oligomeric state of Aβ was significantly stabilized by crocetin. Note that no significant inner filter effect of crocetin should occur in this case due to its negligible absorbance at λ_{ex} (=508 nm) and λ_{em} (=536 nm) used for FlAsH fluorescence measurements. Taken altogether, our results demonstrate that crocetin caused stabilization of Aβ oligomers and prevented their conversion to Aβ fibrils. Moreover, the presence of crocetin significantly preserved A11-positive conformational epitopes of Aβ oligomers.

4. Discussion

Aβ aggregation proceeds from the monomeric state through the oligomeric intermediates, which ultimately form mature amyloid fibrils [1]. Our results demonstrate that crocetin significantly inhibited Aβ fibrillization and destabilized pre-formed Aβ fibril networks. Moreover, crocetin stabilized Aβ oligomers and prevented their conversion to fibrils. Crocetin consists of a conjugated polyene hydrocarbon chain with carboxyl groups attached at both ends. Structurally similar fatty acids containing a hydrocarbon chain and a carboxyl acid at one end were also found to provide similar Aβ oligomer-stabilizing effects at their micellar concentrations [21]. Sodium dodecyl sulfate (SDS), which consists of a 12-carbon tail attached to a sulfate group, also induces formation of stable Aβ oligomers at submicellar concentrations [4,22]. While formation of stable Aβ oligomers from the monomeric state caused by the fatty acids and SDS was driven by immediate secondary structural change of Aβ, for example, from a random coil state to a predominantly β-sheet conformation [4,21], no such structural change was observed when crocetin was added to a solution containing mostly random-coiled Aβ monomers (Fig. S3). The implication is that the mechanism of Aβ oligomer stabilization by crocetin is different from that by fatty acids or SDS. Whether crocetin forms micelles is currently unknown, though micelle formation was not necessary to stabilize Aβ oligomers by SDS [4,22]. Note that multiple pathways exist to form Aβ oligomers [23–25] and not all Aβ oligomeric species appeared to be obligatory intermediates to fibril formation [23,24]. For example, Aβ oligomers found in the presence of fatty acids and SDS represent "off-pathway" oligomeric species which are formed independently of the fibril pathway [4,23]. More comprehensive biochemical and biophysical studies will be required to better understand whether stable Aβ oligomers formed in the presence of crocetin are on- or off-pathway to form fibrils.

Crocetin's negatively charged carboxyl groups and its hydrophobic hydrocarbon chain are likely to interact with positively charged Aβ amino acids and hydrophobic Aβ sequences, respectively. There are three positive residues, Arg 5, Lys 16 and Lys 28 in Aβ at neutral pH (Fig. 1A). We speculate that crocetin can interact with Lys 16 or Lys 28 rather than Arg 5, and the Aβ hydrophobic C-terminal domain (Ile31–Val40) to stabilize Aβ oligomers for the following reasons: (1) the Aβ N-terminal sequence (Asp1–Glu11) is non-amyloidogenic and not aggregation-prone [26–29]; (2) the Aβ C-terminal domain is critical in formation of stable Aβ oligomers [4,22,23,30]; (3) the conformational states in or around Aβ residues 20–30 can determine, at least in part, whether Aβ forms oligomers or fibrils [30–34]. Crocetin may also affect an interfacial arrangement between Aβ monomeric subunits comprising Aβ oligomers, another key factor to determine whether Aβ proceeds along a pathway to form stable oligomers or fibrils [35]. Interactions between crocetin and Aβ may be modulated by varying the length and saturation degree of the hydrocarbon chain of crocetin, as was the case with interactions between fatty acids and Aβ [4,21,30].

Our study suggests that pharmaceutical profiles of crocetin have to be carefully evaluated, despite its other favorable pharmaceutical effects, due to the potential accumulation of Aβ oligomeric forms, a significant risk factor of AD [1,2]. Orally administered crocetin may be absorbed into blood [10] and thus may cause accumulation of Aβ oligomers in the periphery. In addition, crocetin may cross over the BBB [36] and thus induce formation of stable Aβ oligomers in the brain. Interactions between crocetin and Aβ may further be facilitated by a common binding partner, HSA. As such, comprehensive, multi-aspect examination of pharmaceutical effects of crocetin and pathophysiological relevance of crocetin-stabilized Aβ oligomers will be required for careful evaluation of therapeutic benefits of crocetin administration.

Recent studies demonstrate that there exist various Aβ oligomers differing in conformation [34,37,38]. Conformational dissimilarity is usually discerned by a conformation-specific antibody, such as A11. Note that not all Aβ oligomers are recognized by A11 [34,37,38]. A11-positive Aβ oligomers have been found in AD brains [3], but not in normal age-matched brains [3], indicating the pathological relevance of these oligomers. Molecular level structures of Aβ oligomers displaying A11-positive conformational epitopes thus have significant scientific and clinical implications,

but are currently unavailable, at least in part, due to insufficient conformational stability of these oligomers [39]. Our results suggest that crocetin may serve as an efficient stabilizer of A11-positive Aβ oligomers, allowing for their extensive structural studies at the molecular level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.025.

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