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An Investigation on the Interaction of Various Natural Products with the Amyloid- β Peptide; Betulinic Acid Causes Rapid Amyloid- β Fibril Formation at the Expense of Soluble Oligomers

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The University of Southern Mississippi

AN INVESTIGATION ON THE INTERACTION OF VARIOUS NATURAL
PRODUCTS WITH THE AMYLOID- β PEPTIDE; BETULINIC ACID CAUSES
RAPID AMYLOID- β FIBRIL FORMATION AT THE EXPENSE OF SOLUBLE
OLIGOMERS

by

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A Thesis

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ABSTRACT

In this report we investigate the interaction of the natural compounds asiatic acid (AA), asiaticoside (AS), umbelliferone (UM), and betulinic acid (BA) with the amyloid- β (A β) peptide, the aggregation of which is implicated to be the major pathogenic event in Alzheimer's Disease (AD). Screening of compounds for ability to affect A β aggregation was performed via ThT fluorescence. Only BA showed consistent deviation from controls, causing virtually instantaneous formation of large amounts of fibrils in a concentration-dependent fashion. Turbidity assays ensured the aggregation monitored via ThT was not experimental aberration. Kinetics utilizing ThT and turbidity indicated that BA causes immediate exponential aggregation before leveling out within 15 min. SEC demonstrated the decrease in monomer caused by BA within 24 h and indicated that BA led to a decrease in oligomeric species. Sedimentation assays confirmed that large amounts of fibrils were formed in 24 h. Circular dichroism (CD) showed that BA caused instantaneous conversion of A β monomer from random coil to β -sheet, and that the maximal difference in secondary structure from controls was seen at 24 h. Immunoblots confirmed 24 h fibril formation, and also indicated that BA prevented the formation of oligomeric species found in control samples. Fluorescence anisotropy confirmed that BA binds to A β with a K_D^{app} of 11.02 ± 2.01 μ M. We conclude that AA, AS, and UM do not directly affect A β aggregation, while BA promotes rapid A β fibrilization at the expense of soluble oligomeric species. Because oligomers are the most neurotoxic form of A β , this suggests that BA should be further investigated as a potential therapeutic for AD.

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Introduction

Alzheimer's disease and amyloid- β

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder, and is the most common among all neurodegenerative diseases. It is characterized by such symptoms as acute memory loss and cognitive decline. These symptoms are caused by the deposition of amyloid- β (A β) peptide in the brain, forming what are known as senile plaques, and causing neuronal death, synaptic dysfunction, and loss of brain mass¹. These proteinaceous aggregates of A β are thought to be the main pathogenic species responsible for the neuronal loss and toxicity in AD brains. The generation of this hypothesis was driven largely by the recognition that the gene encoding A β is localized on chromosome 21 and that individuals with Down Syndrome – Trisomy 21 – invariably develop AD pathology^{2,3}. The hypothesis was further strengthened when several familial forms of AD were traced to mutations in the gene encoding amyloid precursor protein (APP)⁴. APP is a membrane-bound protein that is sequentially cleaved by two membrane-bound proteases (β - and γ -secretase, respectively) extracellularly to generate A β ⁵. This process is not inherently pathogenic, but changes in the cleavage cascade as a result of aging or mutations lead to the aggregation of A β . In particular, mutations in the presenilin genes (*PS1* and *PS2*) have been shown to increase A β aggregation by altering the γ -secretase mediated cleavage to produce a residue of 42 amino acids (A β 42), rather than the usual 40 (A β 40)^{5,6}. A β 42 has a higher tendency for self aggregation and is the more neurotoxic of the two forms of A β ⁷.

A β aggregation and oligomer toxicity

The process of A β aggregation begins when monomeric A β spontaneously converts from random coil to β sheet secondary structure. This conversion allows multiple monomers to come together to form cross β sheet structures. Individual molecules of A β display a β strand, turn, β strand motif, and adjacent molecules of A β associate via intermolecular interaction of the R groups between the N-terminal β strand of one molecule and the C-terminal β strand of the other⁸. In a process analogous to crystal growth, these small, oligomeric clumps of A β serve as nuclei for the recruitment of other A β monomers. This leads to a logarithmic growth phase, in which monomeric and oligomeric species go on to form insoluble fibrils, which in turn deposit in the brain as senile plaques. Much early research focused on the insoluble fibers and senile plaques as the pathogenic species of AD³. However, more recent investigations suggest that the soluble, intermediate oligomers are the primary neurotoxic agents responsible for the symptoms of AD^{9,10}. These oligomers exist in equilibrium with the large plaque deposits, which serve as reservoirs of inactive protein³. Microinjection of isolated oligomers in mice caused inhibition of long term potentiation (LTP), which is thought to be the molecular mechanism of memory formation, showing that oligomers exhibited toxicity even in the absence of monomers and insoluble fibrils¹¹. Indeed, the level of cognitive decline and the severity of AD symptoms correlates poorly with the level of insoluble plaque found in the brain¹². In addition, neuroinflammation has been shown to play a key role in the pathology of AD^{13,41}. This inflammation, mediated largely by microglial cells – the immune cells of the brain – has been shown to be triggered by the presence of oligomeric A β species, lending further credence to the hypothesis that oligomers are the primary causative agents of AD¹⁴.

A β aggregation occurs via multiple pathways

Although the obligatory “on-pathway” in which monomers proceed continuously toward fibril formation is certainly a component of the AD disease cascade, recent research has indicated that A β monomer can follow multiple pathways of aggregation, some of which result in kinetically stable oligomers that fail to proceed to insoluble fibrils. Hypothesizing that endogenous interfaces, such as those formed by lipid rafts, influenced the A β aggregation pathway, Rangachari and colleagues showed that A β incubated in the presence of sodium dodecyl sulfate (SDS) micelles formed stable dimers and trimers, while removal of SDS caused these species to shift to unstable, higher molecular weight oligomers¹⁵. Another study found that the presence of non-esterified fatty acids (NEFAs) near to and above their critical micelle concentration (CMC) caused the formation of 12-18mers and 4-5mers, respectively, which remained kinetically trapped in an “off-pathway” as oligomers as long as NEFAs remained in the solution¹⁶. Removal of fatty acids allowed the oligomeric species to go on to form fibrils. However, even in the presence of NEFAs, an equilibrium exists between monomers, oligomers, and fibrils, suggesting that the on-pathway and off-pathway may occur simultaneously. It is possible that shifting this equilibrium *in vivo* could cause a decrease in the amount of highly toxic oligomeric species in favor of monomers or fibrillar aggregates.

AD drug development

Development or discovery of molecules that inhibit the aggregation of A β has been a major pursuit of AD research. A number of small molecule inhibitors, including some natural products, are reported to disrupt A β aggregation *in vitro* and reduce toxicity. Perhaps the most well known of these molecules is curcumin, a component of

turmeric^{17,18}. These molecules are thought to destabilize the A β fibrils and/or to significantly reduce the rate of aggregation, but the precise mechanism of inhibition is not yet clearly understood. Developing A β aggregation inhibitors poses several challenges: *a*) it is extremely difficult to control of the stage of inhibition during the aggregation process, and *b*) inhibition of fibril formation may result in an increase in the concentrations of the intermediate oligomers along the aggregation pathway, which may prove to be more detrimental, as smaller soluble oligomers are known to more toxic than fibrils. Therefore, an ideal way of developing therapeutically viable aggregation inhibitors would be to trap A β at the monomeric level, since monomers are not toxic. However, it is difficult to design molecules against intrinsically disordered, monomeric A β due to its high conformational flexibility and lack of structurally well-defined recognition motifs.

Here, we report the interaction of various natural products with A β 42 peptide and their effect on the latter's aggregation process. Many natural products derived from plants have unique pharmacological properties that can be beneficial in the drug development process. Ancient forms of therapy, such as the 'Ayurvedic' type practiced in India, have extensively used plant products in the form of herbal concoctions for the treatment of numerous disorders, including those of the CNS. In this work, we report on the interactions of the natural compounds asiatic acid (AA), asiaticoside (AS), umbelliferone (UM), and betulinic acid (BA).

Asiatic acid and asiaticoside

C. asiatica has been traditionally used in Ayurvedic medicine to treat cognitive impairment and decline in a concoction comprising *C. asiatica* and three other herbs to be taken with milk¹⁹. The remedy is said to improve memory, prevent dementia, and fend off the negative effects of aging. It may also have anti-inflammatory effects.

In fact, *C. asiatica* has been investigated in several studies related to cognitive impairment or decline. Haleagrahara and Ponnusamy investigated the neuroprotective effect of an extract of *C. asiatica* on reactive oxygen species in rats that had been induced to have Parkinson's disease²⁰. As in AD, the pathology of Parkinson's disease involves the creation of free radicals and the oxidative damage done by these radicals on the nerve tissues. In rats who were exposed to a compound chosen to induce oxidative damage and *C. asiatica* extract, levels of lipid hydroperoxides and protein-carbonyl content decreased while levels of total antioxidants and antioxidant enzyme levels increased, suggesting *C. asiatica* extract is capable of protecting the brain from free-radical induced damage. A study by Tiwari et al. investigated the effect of oral administration of *C. asiatica* extract on elderly patients in a hospital to determine its effect on mild cognitive impairment (MCI)²¹. After treatment, the participants exhibited an increase in mini mental status examination (MMSE) scores, a decrease in mean diastolic blood pressure, an increase in appetite, an improvement in their sense of well being, a decrease in insomnia, and a decrease in sensations of numbness and burning, perhaps due to anti-inflammatory effects of *C. asiatica* extract.

Figure 1. Amino acid sequence of A β 42 and A β 42 mutants; structures of compounds used in this study.

Patil et al. investigated the effect of asiatic acid, a component of the *C. asiatica* extract, along with withanolide A, another plant extract, on multiple targets associated with APP processing and the clearance of A β ²². It was found that asiatic acid both upregulated the activity of the ADAM10 enzyme, which is involved in non-amyloidogenic processing of APP, and downregulated the activity of BACE1, a rate-limiting enzyme involved in the production of A β from APP. Asiatic acid was also investigated by Mook-Jung et al. for its protective effects against A β neurotoxicity²³. The researchers used lab-grown cells and applied asiatic acid and two other asiaticoside derivatives to separate cell cultures. All three compounds were found to reduce cell death induced by hydrogen peroxide and to lower concentrations of free radicals within the

cells, with asiatic acid having the strongest effect. In addition, neither asiatic acid nor the other two compounds showed any toxicity or reduction in function of hippocampal cells. Another related study investigated the effect of eight semi-synthetic asiatic acid derivatives for their neuroprotective effect²⁴. This study found asiatic acid to be the most effective protector against neurotoxicity, demonstrating 97% protection against direct A β toxicity when A β was added to a culture of cells. In addition, a study by Boopathy et al. investigated asiatic acid's effect as an acetylcholinesterase inhibitor²⁵. Elevated acetylcholinesterase activity is a characteristic feature of AD. Asiatic acid was applied to hippocampal cell lines, and the cells were then assayed for acetylcholinesterase activity and for any potentially toxic effect of asiatic acid. Asiatic acid was found to be a very effective inhibitor of acetylcholinesterase while exhibiting no toxic effects on the hippocampal cells.

While all of these lines of research directly relate to AD and A β toxicity, we were unable to locate any studies directly investigating the effect of asiatic acid upon A β aggregation. Because of its clear potential to reduce A β toxicity, we have decided to investigate it herein, along with asiaticoside, a closely related compound also found in extracts of *C. asiatica*. We observed, however, that in spite of the established neuroprotective effect of *C. asiatica* extract and its derivatives, neither asiatic acid nor asiaticoside exhibited significant fibril inhibition *in vitro*.

Umbelliferone

Coumarin is a chemical family within the benzopyrene class. Among the coumarins is the natural product umbelliferone, which is a component of various Ayurvedic remedies for cognitive impairment⁴⁴. It is hydrophobic, small, and should therefore be able to

penetrate the blood-brain barrier. Because of these factors, we included umbelliferone among our list of compounds to be tested.

Betulinic acid

BA is another natural product and has been used therapeutically both in Ayurveda and modern medicine. BA is a lupane-type triterpenoid (Figure 1) and a major component in many different plants including *Bacopa monniera*, also known as ‘Bhrami,’ which grows in south Asia²⁶. Extracts of *Bracopa monniera* are used in Ayurvedic medicines for the treatment of CNS disorders and mental function²⁷. They are also thought to improve higher order cognitive processes that are critically dependent on the input of information from our environment such as learning and memory²⁸. In addition, the bark of the white birch, *Betula alba*, which contains BA, has been extensively used by native Americans to treat intestinal disorders. Furthermore, BA has also shown great promise as a potential therapeutic for a variety of cancer types, including neuroectodermal tumors, and is currently in Phase II clinical trials for the treatment of dysplastic nevi with moderate to severe dysplasia. Critically, the hydrophobic nature and small size of BA should enable it to cross the blood-brain barrier, which makes it a compound suitable for the treatment of CNS disorders²⁶.

Therefore, in this report, we investigate whether BA can interact with A β 42 *in vitro*. We observed that BA binds to A β 42 and augments its rate of fibril formation in a concentration-dependant manner. In addition, this augmentation appears to occur in such a way as to bypass neurotoxic, soluble intermediates along the fibril aggregation pathway.

Materials and Methods

Materials

A β 42 was synthesized at the Peptide Synthesis Facility at the Mayo Clinic (Rochester, MN) using routine Fmoc chemistry. MALDI-ToF mass spectrometry revealed > 90% purity of both peptides. SDS, bovine serum albumin, asiatic acid, asiaticoside, umbelliferone, scopoletin, betulinic acid, and thioflavin T were procured from Sigma (St. Louis, MO). All other chemicals were obtained from VWR Inc.

Preparation of A β 42 monomers

Lyophilized stocks of synthetic A β 42 were stored at - 20 °C, desiccated. Prior to the experiments, any pre-formed aggregates that may have been present were removed via size exclusion chromatography (SEC) as previously reported¹⁶. Briefly, 1-1.5 mg of the peptide were dissolved in 0.5 ml of 30 mM NaOH, and allowed to stand at room temperature for 15 min before loading onto a Superdex-75 HR 10/30 size exclusion column (SEC) (GE Life Sciences) attached to an AKTA FPLC system (GE Healthcare, Buckinghamshire). The column was pre-equilibrated in 20 mM Tris-HCl (pH 8.0) at 25 °C and was run at a flow rate of 0.5 mL/min. One minute fractions were collected. Concentrations of the purified fractions were estimated by UV-Vis spectroscopy on a Cary 50 spectrophotometer (Varian Inc) using a molar extinction coefficient (ϵ) of 1490 cm⁻¹ M⁻¹ (www.expasy.org), corresponding to the single tyrosine residue within A β 42. Peptide integrity after SEC was again confirmed by MALDI-TOF mass spectrometry, which yielded a single peak corresponding to a monoisotopic molecular mass of 4516.31 Da in a good agreement with the calculated mass of 4514.13 Da. A similar purification scheme was also adopted for mutant A β 42 F19W. Monomeric A β 42 fractions were

stored at 4 °C and were used within 3 to 4 days of purification in all experiments to avoid the presence of pre-formed aggregates in our reactions.

A β 42 oligomer generation

A β 42 12-18mers were generated by following the method reported previously¹⁶. Briefly, 50 μ M A β 42 was incubated with 5 mM lauric acid at 37 °C for 48 h. The sample was then fractionated using a Superdex-75 SEC column and the fractions 17 and 18 were collected for further use.

A β aggregation reactions

Unless otherwise noted, reactions and measurements were done at room temperature, and samples were stored at 37 °C. Reactions were initiated in 0.5 mL siliconized eppendorf tubes by incubating freshly purified A β 42 monomer in appropriate conditions in buffer without agitation. Aggregation parameters were obtained by monitoring the reaction with thioflavin T (ThT) fluorescence and fitting fluorescence data points to the sigmoidal curve in eq. 1 using Origin 7.0. In this equation t is time, a and b are fixed parameters, and $t_{0.5}$ is the time to reach half-maximal ThT fluorescence. Lag times were

equal to $t_{0.5} - 2b$ for each curve.

Fluorescence spectroscopy

ThT fluorescence (F) was monitored in a 1 cm quartz microcuvette with a Cary Eclipse spectrometer (Varian Inc) after 15-fold dilution of A β 42 samples into 5 mM Tris-

HCl (pH 8.0) containing 10 μ M ThT. Continuous measurements of F were taken for 1 min with the excitation and emission wavelengths fixed at 450 and 482 nm respectively and both slits set at 10 nm. The fluorescence blanks were subtracted from the respective data and the average F value was determined.

Turbidity

Turbidity was monitored in a 1 cm quartz microcuvette using a Cary 50 UV-Vis spectrophotometer (Agilent Technologies). At the given time points, 70 μ L of undiluted sample was placed into the cuvette and absorbance was measured at 400 nm using the 'Simple Read' program provided by the manufacturer in triplicates with 30 s delays.

Kinetics

The initial, or zero-hour, kinetics of an A β 42 aggregation reaction were obtained in a similar fashion to the aggregation reactions already described. For kinetics, however, the reaction was prepared with all components except for the freshly purified A β 42 monomer. For fluorescence, the fluorescence spectrophotometer was set as above, except with a 15 min collection time. Immediately before measurement, the A β 42 monomer was mixed with the sample, and 5 μ L of sample was combined with 70 μ L of ThT in the quartz cuvette. The sample was quickly mixed and inserted into the spectrophotometer for data collection. For UV kinetics, the same sample preparation technique was used, and absorbance measurements were taken on the Cary 50 UV-Vis spectrophotometer using the 'Kinetics' program provided by the manufacturer.

Size exclusion chromatography

Quantitative estimates of aggregation reaction were obtained using a Superdex-75 HR 10/30 size exclusion column (GE Life Sciences) on an AKTA FPLC system. Aliquots of sample from the aggregation reactions were centrifuged and the supernatant (100 μ L) was loaded onto the SEC column. The samples were then subjected to fractionation in 20 mM Tris-HCl, pH 8.0 at a constant flow rate of 0.5 mL/min and the elution was monitored at the wavelength of 215 nm.

Polyacrylamide gel electrophoreses (PAGE) and immunoblotting

Samples were dissolved in loading buffer (1x Laemmli buffer) containing 1% SDS, applied without heating to 4-12% NuPage gels (Invitrogen) containing bis-Tris, and resolved in 2-(N-morpholino)ethanesulfonic acid (MES) running buffer with 0.1% SDS. Dye-linked MW markers (Blue Plus2 Prestained Standards, Invitrogen) were run in parallel for calibration. Gels were electroblotted onto 0.45 μ m immobilon nitrocellulose membranes (BioTraceTM NT, Life Sciences Inc). Blots were boiled in a microwave oven in PBS for 2 min and were blocked overnight with 1X PBS containing 5% nonfat dry milk and 0.1% tween-20 before probing (1-2 h) with 1:1000-1:2500 dilutions of Ab9 monoclonal antibody, which detects amino acid residues of A β (1-16). Blots were then incubated with anti-mouse horseradish peroxidase (HRP) conjugate and developed with ECL reagent (Thermo Scientific).

Circular dichroism (CD)

CD spectra were obtained in the far UV region with a Jasco J-815 spectropolarimeter (Jasco Inc, Easton, MD). Aliquots of samples containing A β 42 (25 μ M) were placed in a 0.1 cm path-length quartz cuvette (Hellma) and were monitored in continuous scan mode (260-190 nm). The acquisition parameters were 50 nm/min with 8 s response time, 1 nm bandwidth and 0.1 nm data pitch, and data sets were averaged over three scans. All data were collected in duplicate. Spectra of appropriate blanks were subtracted from the data sets as indicated. The corrected, averaged spectra were smoothed using the 'means-movement' algorithm with a convolution width of 25 using the Jasco spectra analysis program.

Fluorescence anisotropy

Binding experiments were carried out by fluorescence anisotropy experiments using an F19W-A β 42 mutant. The excitation and emission wavelengths were fixed at 280 and 352 nm respectively with a spectral bandwidth of 30 nm (10 nm Ex and 20 nm, Em). The ADL program on spectrometer was used and the G-factor was calculated for each titration using A β 42 F19W as the fluorophore. Each titration point was measured in quadruplets after a brief equilibration time of 1 min, and the data points were averaged. The data was converted to anisotropy (r) values using the 'Advanced Read' program provided by the manufacturer. The data were fit into the following non-cooperative single site binding equation using Origin 7.0 (Eq 2), where r_0 and r_s are anisotropy values in the

absence and saturated levels of the ligand (BA), respectively, while L_t and P_t are the respective total ligand and A β 42 concentrations.

Results

AA, AS, and UM fail to inhibit A β 42 fibrilization

Using ThT fluorescence as described above, we investigated the effect of asiatic acid, asiaticoside, umbelliferone, and scopoletin on A β 42 fibril formation. ThT is an extrinsic dye that is known to show fluorescent properties upon binding to amyloid aggregates²⁹ and hence, it is used as an indicator to monitor A β fibril formation or aggregation.

Figure 1A shows an aggregation profile of each compound incubated at a fourfold molar excess to A β 42. Although datasets from replicate incubations could not be averaged due to the slight variation in purity of A β 42 monomer, the chosen dataset is typical of all aggregation profiles obtained. All compounds were dissolved in DMSO, except for AS, which was soluble at 300 μ M in water. Appropriate controls were included for each solvent. We incubated 25 μ M A β 42 with 100 μ M BA, AA, AS, or UM in 20 mM TRIS, pH 8.0. Appropriate controls were also generated, with the same percentage of DMSO (5%) included in the DMSO control as was present in the final incubations of BA, AA, and UM. The aqueous control simply contained 25 μ M A β 42 and 20 mM TRIS at pH 8.0. Betulinic acid (\blacktriangle) displayed an instantaneous increase in fluorescence, which was sustained throughout the incubation period. Asiatic acid (Δ) and the DMSO control (\bullet) displayed a very similar aggregation profile, reaching a similar maximum level of fluorescence with lag times of approximately 24 h. Earlier incubations had shown umbelliferone to similarly diverge very little from the DMSO control (data

not shown). Asiaticoside (\square) displayed no major divergence from the aqueous control (\blacksquare) in either lag time or maximum fluorescence in this particular incubation, but in other replications it seemed to cause slight inhibition of fibril formation (data not shown).

In order to further investigate the effect of these compounds on aggregation of A β 42, we incubated them at sixfold excess with E22G-A β 42 mutant, in which the glutamate residue at position 22 was replaced with glycine. E22G-A β 42 typically aggregates faster than wild type, and investigating the interaction of compounds with A β mutants can help to determine which portions of the A β peptide are involved in the interaction. Figure 1B shows that BA (\circ) again caused an increase in fluorescence with no noticeable lag time, which remained high throughout the incubation. UM (\blacktriangle) and AA (\square) had very similar fluorescence to the DMSO control (\bullet) for the duration of the experiment, and AS (Δ) again displayed very little divergence from the aqueous control (\blacksquare).

Since AS and BA were the only compounds to show any divergence from their controls, we incubated them at a tenfold molar excess to A β 42 in an attempt to make their effect more apparent. 25 μ M A β 42 was incubated with 250 μ M BA or AS in the same conditions as previously described, with appropriate controls. Figure 1B shows that betulinic acid (\circ) caused an even greater increase in total fluorescence with no noticeable lag time, compared to the DMSO control (\bullet). On the other hand, asiaticoside (Δ) did not display any inhibitory effect compared to the aqueous control (\blacksquare) at tenfold excess, suggesting that the slight inhibition seen earlier was an experimental aberration. However, the effect of BA was increased with increasing dosage, a phenomenon which we chose to further investigate and characterize.

BA rapidly augments A β 42 fibril formation in a concentration dependent manner

We explored the effects of BA on A β 42 aggregation by ThT fluorescence and turbidity assays. Since BA shows poor solubility in aqueous buffers, we performed our experiments in two different solvents: dimethyl sulphoxide (DMSO) and ethanol (EtOH). As described in a subsequent section, we used EtOH as a solvent to facilitate circular dichroism (CD) measurements, as DMSO is not well tolerated in CD. It was therefore essential to monitor aggregation in both solvents to ensure that the interaction between betulinic acid and A β 42 was maintained.

We incubated 25 μ M of freshly purified, seed-free monomeric A β 42 with increasing

Figure 2. ThT Aggregation Profiles. ThT fluorescence over time for incubations of A) fourfold molar excess of BA (\blacktriangle), AA (Δ), and AS (\square) incubated with A β 42, along with DMSO control (\bullet) and TRIS control (\blacksquare); B) sixfold molar excess of BA (\circ), UM (\blacktriangle), AA (\square), and AS (Δ) incubated with E22G-A β 42, along with DMSO control (\bullet) and TRIS control (\blacksquare); and C) tenfold molar excess of BA (\circ) and AS (Δ), along with DMSO control (\bullet) and TRIS control (\blacksquare).

Figure 3. ThT Aggregation Profiles. ThT fluorescence over time for incubations of A) fourfold molar excess of BA (\blacktriangle), AA (Δ), and AS (\square) incubated with A β 42, along with DMSO control (\bullet) and TRIS control (\blacksquare); B) sixfold molar excess of BA (\circ), UM (\blacktriangle), AA (\square), and AS (Δ) incubated with E22G-A β 42, along with DMSO control (\bullet) and TRIS control (\blacksquare); and C) tenfold molar excess of BA (\circ) and AS (Δ), along with DMSO control (\bullet) and TRIS control (\blacksquare).

concentrations of BA (from sub-stoichiometric ratio to four-fold excess) at 37 °C. Since the stock solutions (1 mM) of BA were prepared in 100% DMSO or EtOH, incubations of varying A β :BA stoichiometry resulted in different final concentrations of DMSO or EtOH in the sample. A ratio of 1:4 A β ₄₂:BA contained 5%, 1:2 contained 2.5%, 1:1 had 1.25% while 1:0.5 contained 0.625% of DMSO or EtOH. Therefore, appropriate A β ₄₂ samples in similar solvents but in the absence of BA were used as controls (Figure 3B and 3D). Aliquots of the incubated samples at the given time points were subjected to fluorescence measurement upon addition of ThT buffer as shown in Figure 3A and 3C for DMSO and EtOH, respectively. The data were fit using a sigmoidal equation (Eq. 1 in Materials and Methods) to obtain lag-time information. Incubation in DMSO showed an increase in A β ₄₂ aggregation rate as observed by both the increase in the fluorescence intensity and the decrease in lag times as compared to the controls. The difference plots shown in Figure 3E show the dose-response effect in which 1:4 ratio (\square) had the highest rate of aggregation, followed by 1:2 (\blacktriangle), 1:1 (\circ) and 1:0.5 (\blacksquare). The four-fold excess of BA seemed to show immediate and sharp increase in ThT fluorescence intensity with no noticeable lag-time (\square in Figure 3A and E). Even a sub-stoichiometric ratio of 1:0.5 of A β ₄₂:BA (\blacksquare in Figure 3E) resulted in augmentation of the aggregation rate as indicated by the fluorescence increase after ~50 h of incubation. Similarly, incubations in EtOH also resulted in augmented A β ₄₂ aggregation rates with lag times similar to those observed with DMSO (Figure 3C). However, control incubations in EtOH but in the absence of BA also resulted in increase in A β ₄₂ aggregation rates. Nevertheless, the difference plots of the incubations in EtOH shows that there is a net increase in the aggregation rates that is prominent with 1:4 stoichiometry (Figure 3F). It is noteworthy that the incubation at a 1:4 molar ratio showed a rapid increase in ThT fluorescence in

both solvents within the first 24 h (Figures 3E and F). Reproducible results were obtained multiple times for both solvents. However, due to the slight variation in A β monomer purity, the lag-times varied, making it difficult to average the data sets. Nevertheless, overall reaction profiles remained identical to the one shown in Figure 2.

Since we observed maximal effect (in ThT measurements) with a fourfold molar excess of BA, we explored only the sample of 25 μ M A β 42 incubated with 100 μ M BA in DMSO at 37 °C for the turbidity assay, since its primary purpose was to confirm the validity of ThT measurements. We monitored aggregation by measuring the absorbance at 400 nm in a UV-Vis spectrophotometer as previously described³⁰. A β 42 incubated with BA showed a rapid increase in turbidity within 24 h of incubation and continued to remain high, consistent with the ThT experiments (\circ ; Figure 4A). The control A β 42 sample in the absence of BA also displayed a similar trend; however, the absorbance intensity was far lower (\bullet ; Figure 4A). The difference curve showed a rapid increase during the initial 24 h that remained fairly stable for the remainder of the incubation period (\blacktriangle ; Figure 4A). The difference curve was again similar to that for the ThT fluorescence experiment (Figure 3A).

With 1:4 stoichiometric incubations we observed a sharp and apparently instantaneous increase in both fluorescence and absorbance levels (Figures 3A and 4A, respectively), and we wanted to ensure it was not an experimental aberration. Therefore, we monitored the aggregation process immediately upon coincubation of BA and A β 42. As shown in Figure 4B, both ThT fluorescence and absorbance showed a rapid kinetic process with similar exponential growth rates, suggesting a rapid and considerable increase in aggregation rate induced by BA.

Figure 3. Dose-dependent augmentation of A β 42 aggregation by BA.

Aggregation was measured via ThT fluorescence over time. Incubations comprised of 25 μ M A β 42 in 20 mM Tris, pH 8.0 at 37 °C with increasing amounts of BA dissolved in DMSO; A) 12.5 μ M (■), 25 μ M (○), 50 μ M (▲), 100 μ M (□). B) Controls containing DMSO concentrations corresponding to 12.5 μ M, 25 μ M, 50 μ M, and 100 μ M BA incubations respectively shown in A: 0.625% (▲), 1.25% (○), 2.5% (□), and 5% (■). C) Incubations comprising buffered 25 μ M A β 42 with increasing amounts of BA dissolved in EtOH; 12.5 μ M (■), 25 μ M (○), 50 μ M (▲), and 100 μ M (□). D) Controls containing varying EtOH concentrations; 0.625% (■), 1.25% (○), 2.5% (▲), and 5% (□). E) Difference plot of BA dissolved in DMSO (A) and DMSO controls (B). The control fluorescence at each time point was subtracted from the fluorescence of the BA samples to generate the plot, which shows the molar stoichiometric incubations, 1:0.5 (■), 1:1 (○), 1:2 (▲), and 1:4 (□). F) Similar difference plot of BA dissolved in EtOH (C) and EtOH controls (D), which shows the molar stoichiometric incubations, 1:0.5 (■), 1:1 (○), 1:2 (▲), and 1:4 (□). The A β :BA stoichiometry is indicated by dotted arrows in E and F.

Figure 4. Other assays also suggest rapid A β fibrillation induced by BA. A) Turbidity assay was performed by measuring the absorbance at 400 nm for buffered 25 μ M A β 42 in 5% DMSO at 37 °C in the presence of 100 μ M BA (\circ) or in the absence of BA (\bullet). The difference plot is also shown (\blacktriangle). B) Kinetics of the incubations in (A) was monitored immediately after incubation ThT fluorescence (grey line) or absorbance at 400 nm (black line). C) The samples in (A) were subjected to fractionation via SEC after 24 h of incubation. The samples were subjected to centrifugation at 19000g for 15 minutes and the supernatants were loaded on to the Spersdex-75 column. The sample containing BA is shown as black line whereas the control sample in the absence of BA is shown as a dotted line. The monomers eluted at fractions 22-26, while the aggregate peak near the void volume (V_o) between 15 and 17 (inset). D) Sedimentation assay was performed by taking an aliquot of the co-incubation of A β 42 with four-fold molar excess of BA was measured for ThT fluorescence after 24 h (T). In parallel, another aliquot of the same reaction was subjected to centrifugation at 19000g for 15 min. The supernatant thus obtained was measured via ThT fluorescence (S). Three such data sets were averaged.

Sedimentation and SEC assays also indicate rapid A β 42 fibrilization in the presence of BA.

In order to quantify the proportion of monomeric A β 42 being converted to insoluble fibrils, we monitored the aggregation process by using sedimentation and size exclusion chromatography (SEC). We monitored the depletion of monomers when A β 42 was incubated with BA in comparison to a control in the absence of BA using a

Superdex-75 SEC column. Since we observed the maximum difference between experimental and control samples after 24 h of incubation with a fourfold molar excess of BA (Figure 3A and 4A), we decided to fractionate a sample containing 25 μ M A β 42 and 100 μ M BA, as well as a 25 μ M A β control, after 24 h incubation at 37 °C. First, we centrifuged each sample at 19,000g for 15 minutes in order to sediment any pre-formed insoluble fibrils that may have interfered with the SEC column. The supernatant was then loaded on to the column for fractionation. The A β 42 control incubation in the absence of BA showed a distribution containing mainly the monomeric peak, which eluted in fractions, 24 and 25 along with a minor amount of aggregated material eluting near the void volume (V_0) of the column (Figure 4C; dotted line). The A β 42 sample in the presence of BA showed a similar fractionation profile (Figure 4C; solid line); however, the amount of monomer was far less than that of the control sample. This suggests that the presence of BA promotes rapid fibril formation, leading to a concomitantly decrease in the amount of monomers. Furthermore, it is interesting to note that the sample with BA also contained some aggregates that eluted near the void volume (Figure 4C; inset). However, the amount of the aggregated peak was less than that observed for the control sample. Since these aggregates were non-fibrillar, soluble intermediates (as the fibrils had been spun out prior to fractionation), this observation suggests that BA promotes insoluble fibril formation at the expense of soluble aggregates. Finally, in order to ensure that the aggregates formed in the presence of BA are indeed fibrils, we performed a sedimentation assay. After 24 hours of incubating 25 μ M A β 42 with BA in fourfold excess, we measured ThT fluorescence as previously described. We then spun the sample at 19,000g for 15 minutes and measured the ThT fluorescence of the supernatant. As shown in Figure 4D (grey bars), only ~30 % of fluorescence was observed after

sedimentation, suggesting a large amount fibril formation within 24 h of incubation. Furthermore, mass spectrometry analysis of the supernatant and pellet from the co-incubated sample after multiple spins, rinsing the pellet each time, revealed that all BA was associated with the fibrils and none was observed in the supernatant (data not shown), suggesting that BA is incorporated or tightly complexed with the fibrillar structure. Collectively, these data suggest that BA was able to rapidly promote the formation of insoluble A β 42 fibrils from monomeric species and bypass soluble oligomeric intermediates in doing so.

Oligomers are absent in co-incubations of A β 42 and BA

We also subjected the samples incubated in Figures 3A and B to electrophoresis and immunoblotting to see whether the results complemented the experiments described above. Shown in Figure 5 are immunoblots of samples of A β 42 with a

fourfold excess of BA prepared in the same fashion as those in Figure 3A, along with appropriate controls. Clearly,

the control sample in the absence of BA showed no high molecular weight bands for 24 h

Figure 5. A β 42 aggregation in the presence of BA monitored by immunoblots. Samples containing 25 μ M A β 42 were incubated alone (control) or with 100 μ M BA (+BA) at 37 °C. The aliquots of the sample was taken at the indicated time points and were run on a SDS-PAGE gel followed by western blotting and immunodetection. The lanes T & S represent total and supernatant (after spinning the sample at 19000g for 20 min to remove fibrils) respectively, after 0, 24 & 48 h of incubation.

(Figure 5A: 24h). After 48 h, aggregate bands of ~100 kDa were apparent, along with some high molecular weight bands that failed to enter the gel consistent with fibrils (F) (Figure 5A: 48 h). In contrast, samples with BA showed a fibril band (F) within 24 h of incubation. More importantly, the supernatant after centrifuging the sample at 19000g for 15 minutes failed to show the high molecular band (Figure 5B; 24 h, S), confirming that these bands correspond to fibrils. A similar pattern was observed after 48 h of incubation (Figure 5B; 48 h). Although the control sample showed similar bands at 48 h, they were also present in the supernatant, suggesting the bands may correspond to non-fibrillar, 'non-pelletable' forms of aggregates. These results confirm that BA is able to promote insoluble fibril formation from A β 42 monomer within 24 hours and also suggest that this occurs in a manner that bypasses the formation of soluble, pre-fibrillar aggregates.

BA induces rapid changes in A β 42 secondary structure.

Figure 6. Secondary structure changes in A β 42 upon incubation with BA. Co-incubation reactions containing four-fold molar excess of BA in EtOH similar to those shown in Figure 2C were monitored using far-UV CD (solid lines) along with control in the absence of BA (dashed lines) at the indicated time points (A-D). E) The difference plot obtained by subtracting the molar ellipticity values at 216 nm of the control sample from the sample containing BA (■), which was overlayed with the difference plot shown in Figure 2F for 1:4 stoichiometry (○)

The secondary structure changes during the incubation of A β 42 with BA were monitored by far-UV CD spectroscopy. Since DMSO solvent is not compatible with CD measurements, A β 42 was incubated in a fourfold excess of BA in 5% EtOH along with an appropriate control as in Figure 3C and 3D. Both the control sample and the one co-incubated with BA displayed a random coil conformation immediately (10 min) after incubation (Figure 6A). However, the molar ellipticity at 216 nm was slightly more

negative for the sample with BA, suggesting a conformational change toward β -sheet. After 24 h of incubation, the sample containing BA clearly displayed a spectrum with negative minimum at 216 nm and positive maximum at \sim 198 nm, indicating a β -sheet conformation characteristic of A β fibrils (Figure 6B). By contrast, the control sample displayed predominantly a random coil structure (Figure 6B). After 48 h, both the control as well as the sample containing BA showed β -sheet conformation and remained in the same conformation for more than 120 h (Figure 6C and 6D). A comparison of the difference spectra obtained from ThT and CD experiments indicated identical changes (Figure 6E). It is clear that the maximal difference of BA compared to controls was observed during the initial 24 h of incubation, which complements the results obtained from ThT, turbidity, SEC, sedimentation assays, and immunoblots.

BA binds to monomeric A β 42 with sub-micromolar binding affinity.

Finally, we wanted to explore the binding interaction between A β 42 and BA. In order to do so, we used an A β 42 F19W mutant peptide in which the phenylalanine at the 19th position was substituted with a tryptophan residue to facilitate fluorescence anisotropy measurements. Although the wild-type A β contains a tyrosine (Tyr) residue at the 10th position, Tyr is not a good fluorophore and the emission intensities were prohibitively low for anisotropy experiments. A β 40 F19W mutant has been previously observed to exhibit no significant difference in aggregation and fibril formation compared to wild type A β 40³¹. To ensure that there was also no significant difference between wild-type A β 42 and the mutant, we performed ThT experiments both in the presence and in the absence of BA (data not shown). These experiments did not show significant divergence from aggregation profile of wild-type A β 42. Upon titrating BA to

a freshly purified monomeric A β 42 F19W sample, we observed a constant increase in tryptophan anisotropy (r) reflecting binding interaction between the two species (Figure 7; ●). Fitting the data using the non-interacting one-site binding

equation (Equation 2) yielded an apparent dissociation constant ($K_D^{app} = 11.02 \pm 2.01 \mu\text{M}$).

Since BA induces rapid aggregation, especially at high molar ratios, the FA experiments

Figure 7. Binding affinity between A β 42 and BA measured by fluorescence anisotropy. The specific mutant, F19WA β 42 was used as a probe to measure tryptophan anisotropy (r). Aliquots of mixtures containing 5 μM A β 42 and 50 μM BA in 2.5 % DMSO was titrated on a solution containing 5 μM A β 42 in a similar solvent (●). The anisotropy was measured in quadruplets for each titration points that were averaged. As a negative control similar experiment was performed using glycylglycine instead of BA (○). Three independent data sets were averaged and fit with a model for a single site binding (Eq 2).

may not reflect the binding interactions between BA and monomeric A β exclusively. For this reason, we refer to the binding affinity as 'apparent,' K_D^{app} . A dipeptide, glycylglycine (GG) was used as a negative control and did not show any appreciable binding isotherm (Figure 7; ○).

Discussion

The design of therapeutics for any disease is a process that faces many challenges. Synthetic, rationally designed drugs, while having excellent potential for specificity, require the development of an efficient synthetic pathway and extensive testing for toxicity. As the world saw with Thalidomide in the 1970's, even a simple change in stereochemistry can lead to vastly altered biological effects. Natural products offer an attractive alternative to synthetic drugs. They are generally produced in abundant quantities in plants or herbs, the efficacy of their synthesis has been streamlined by millions of years of evolution, and they are often already components of the human diet. In addition, many of these products are already in use in ancient forms of traditional medicine, such as the Ayurvedic system of India. It is entirely likely thorough screening of the natural products used in traditional herbal remedies and concoctions will yield a plethora of active compounds and combinations of compounds that can provide significant benefits to human health.

The compounds tested here are all components of such concoctions, and it is important to note that even if a compound does not directly alter the aggregation of A β , it still has the potential to be beneficial in the treatment of AD. For asiatic acid and asiaticoside, this is almost certainly the case. Although our investigations yielded no direct interaction between the A β ₄₂ peptide and either molecule, they have both been shown by previous researchers to be beneficial in this regard¹⁹⁻²⁴. Umbelliferone has not been extensively researched in connection to AD, and again, although we showed no direct interaction between it and A β ₄₂ *in vitro* aggregation, they may still provide some *in vivo* benefit that our assays did not detect. Betulinic acid proved not to be an aggregation inhibitor, which is the type of compound we initially sought, but it may still mitigate the

AD pathology in some way, especially considering its ability to promote the formation of fibrils at the expense of neurotoxic oligomers, discussed in greater detail below.

The development of A β aggregation inhibitors in particular involves several challenges; a) the difficulty in controlling the stage of inhibition during the aggregation process, and b) the possible increase in the soluble oligomer concentrations upon inhibition of fibril formation may be detrimental, as smaller soluble oligomers are known to be more toxic than fibrils. Therefore, an ideal way of developing therapeutically viable aggregation inhibitors would be to trap A β at the monomeric level, since monomers are not toxic. However, it is difficult to design molecules against intrinsically disordered, monomeric A β due to its high conformational flexibility and lack of structurally well-defined recognition motifs. Considering such difficulties, it is not surprising that few aggregation inhibitors have successfully made it through the clinical trials.

In this context, it is perhaps worthwhile to consider designing molecules that can prevent the formation of neurotoxic oligomers by circumventing these pathogenic species. The results reported here suggest BA is precisely able to perform such a task and showcase the potential use of BA as a therapeutic agent against AD. The mechanism of augmentation of aggregation by BA is not entirely a new observation as previously, compounds such as methylene blue and ellagic acid have also been reported to promote rapid A β ₄₂ fibril formation and inhibit neurotoxicity^{30,32}.

However, the rapid fibrillation induced by BA as reported here, which comes at the expense of toxic soluble oligomers, seems to be exclusive to the fibril formation ‘on-pathway’. As it is increasingly becoming evident that many neurotoxic oligomers can be formed outside the obligatory fibril formation pathway, we explored whether BA is able to circumvent such oligomer formation also. BA failed to induce the formation of fibrils and concomitantly inhibit the formation of 12-18mer oligomers (Figure 8; lanes 1 and 2), which were reported to be formed along an alternate pathway of aggregation called ‘off-pathway’¹⁶. In addition, BA failed to convert isolated 12-18mers to fibrils rapidly as observed with monomers (Figure 8), suggesting that BA is unable to interact with oligomers that are not formed along the fibril formation ‘on-pathway’. These suggest that;

a) BA is extremely potent in promoting rapid fibril formation along the ‘on-pathway’, and b) BA is incapable of interacting with aggregates along alternate pathways of aggregation.

Figure 8. Effect of BA on ‘off-pathway’ oligomers. Lanes 1 & 2 show 25 μ M A β 42 containing 5 % DMSO incubated with 5mM lauric acid alone and in the presence of 100 μ M BA respectively. In parallel, the SEC isolated oligomers were also incubated alone and with 100 μ M BA at 37 °C. The aliquots of the sample was taken at the indicated time points and were electrophoresed and immunoblotted. The lanes T & S represent total and supernatant (after spinning the sample at 19000g for 20 min to remove fibrils) respectively, after 0 & 24 h. The double arrows indicate 12-18mer oligomers of A β 42.

Pharmacologically, the properties of BA are well known, and the compound is being administered for a variety of pathologies. Historically, extracts of *Bracopa monniera* have been used in Ayurvedic medicines for the treatment of CNS disorders and impaired mental function^{27,33}. They are also thought to improve higher order cognitive processes that are critically dependent on the input of information from our environment such as learning and memory³³. In addition, white birch bark, *Betula alba*, which contains BA, has been extensively used by native Americans to treat intestinal disorders. BA is also widely used in modern medicine^{34-39,43}. BA has shown great promise as a potential therapeutic for a variety of cancer types, and, furthermore, BA is also known to inhibit HIV viral replication^{36,40}. Due to the distinct pharmacological advantages of BA as a therapeutic agent along with its unique property of promoting A β fibril formation and especially bypassing toxic oligomers along the ‘on-pathway’ fibril formation as reported here, BA is an excellent therapeutic candidate for AD. Therefore, understanding the effects of BA in *in vivo* systems such as cell culture and animal models is imperative for drug development. Our future experiments are focused on addressing these aspects and will be published later.

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