Identification of Polyphenolic Compounds and Black Tea Extract as Potent Inhibitors of Lipid Membrane Destabilization by Aβ$_{42}$ Aggregates

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Abstract. Amyloid-β (Aβ) aggregation is a recognized key process in the pathogenesis of Alzheimer’s disease (AD). Misfolded Aβ peptides self-assemble into higher-order oligomers that compromise membrane integrity, leading to synaptic degeneration and neuronal cell death. The main aim of this study was to explore whether small-molecule compounds and black tea extract can protect phospholipid membranes from disruption by Aβ aggregates. We first established a robust protocol for aggregating Aβ$_{42}$ peptides into a range of oligomers that efficiently permeabilized small unilamellar liposomes. Next, 15 natural plant polyphenolic compounds, 8 N’-benzylidene-benzohydrazide (NBB) compounds and black tea extract were assessed for their ability to antagonize liposome permeabilization by the Aβ$_{42}$ oligomers. Our data indicates that black tea extract, the flavones apigenin and baicalein, and the stilbene nordihydroguaiaretic acid (NDGA) are indeed potent inhibitors. Taking into consideration the results of all the small-molecule polyphenols and NBB compounds, it can be proposed that a dihydroxyphenyl ring structure, alone or as part of a flavone scaffold, is particularly effective for protection against membrane damage by the Aβ$_{42}$ oligomers. Given the critical role of membrane perforation in the neurodegenerative cascade, these conclusions may guide the design and development of novel therapeutic drugs in AD.

Keywords: Alzheimer’s disease, amyloid-β, black tea, liposomes, N’-benzylidene-benzohydrazide, oligomers, polyphenols

INTRODUCTION

Alzheimer’s disease (AD) is a major economical and social burden in the Western world, being the most common cause of dementia in all age groups and the fourth common cause of death following cardiovascular disease, cancer, and stroke [1]. Clinical manifestations include a worsening cognitive deficit, initially limited to memory alone, but which ultimately interferes with normal social and occupational functioning [2]. Neurodegeneration in AD is relentless and irreversible, and an effective disease-modifying therapy still remains to be developed.

Nevertheless, great strides have been made in trying to understand the cellular and molecular mechanisms that induce AD [3]. In particular, a formidable body of evidence gathered from genetic, biochemical, and transgenic mouse studies, supports the so-called
increased propensity of Aβ postmortem AD brains [6]. Mutations associated with pathological hallmarks found in specific regions of larger protofibrils, and insoluble amyloid fibrils [5] that form extracellular plaque deposits, one of the major pathological hallmarks found in specific regions of postmortem AD brains [6]. Mutations associated with increased propensity of Aβε peptide to form protofibrils correlated nicely with neuronal dysfunction and degeneration in a Drosophila model of AD, thus lending strong support to the view that Aβε aggregation is a critical factor driving neuronal dysfunction in AD [7]. Moreover, accumulating evidence indicates that neurodegeneration and memory loss is actually triggered by the highly toxic soluble oligomeric species of Aβ [8–11]. Indeed, levels of soluble Aβ oligomers correlate well with dementia and AD severity [12–14]. The possible mechanisms underlying the neurotoxic actions of Aβ oligomers are under intensive investigation: interaction with membranes, of both cells and organelles, appears to be a critical first step in the pathway to toxicity [15]. Numerous works have demonstrated that Aβ disturbs properties of artificial and isolated biological membranes, and of plasma membranes in living cells [16–19]. Specifically, Aβ oligomers can incorporate into neuronal membranes to form calcium-permeable channels or pores [20, 21]. The resultant excessive increase in intracellular calcium then sets off a cascade of deleterious sequelae culminating in synaptic degeneration and apoptosis [22–24]. In line with the concept that Aβ-induced membrane perforation is a fundamental mechanism, reduction in ability of Aβ oligomers to bind lipid membranes was associated with decreased toxicity to neuronal cells [25, 26]. Also, small-molecule and peptide blockers of Aβ calcium channels potentially protected neurons from cytotoxicity [27, 28]. Notwithstanding this significant body of evidence, a demonstration that membrane perforation and/or destabilization genuinely occur in vivo, resulting in synaptopathy and neuronal cell death, is still lacking.

Several types of low-molecular-weight chemical compounds have been previously shown to inhibit Aβ oligomer formation in vitro [29–33]. Of these, polyphenols are an important class of beneficial naturally-occurring molecules that are common constituents in human diet and medicinal plants [34]. In most cases, foods contain complex mixtures of polyphenols, which are often poorly characterized. Fruit, tea, and red wine represent the main sources, with daily intake averaging 1 g/day [35]. Polyphenols essentially have several hydroxyl groups on aromatic rings, thereby explaining their high antioxidant activity [36]. In recent years, much attention has been paid to the interaction between polyphenols and cellular membranes, since it has been shown that they can interact with and permeate lipid bilayers [37]. Some studies have proposed that polyphenol molecules interact only with the outer leaflet of bilayers [38], whilst others report penetration of the lipid membrane by intercalation of polyphenols between flexible acyl chains of the phospholipids [39]. In particular, a rigidifying effect on the bilayer consequently hindering permeabilization by an Aβ peptide, has been described [40]. Therefore, the anti-Aβ aggregation effects of polyphenols on the one hand, together with their capacity to protect lipid membranes on the other, make them attractive candidate molecules in AD therapy. As a matter of fact, several recent studies involving dietary supplementation with polyphenolic compounds have consistently shown improvement in cognitive function and AD pathology in transgenic mice [41–45]. Again, recent epidemiological studies suggest that tea, one of the most highly consumed polyphenolic beverages in the world, might be a useful agent in the prevention or treatment of AD/dementia [46–48].

In this study, a fluorescence-based vesicle permeabilization assay was first established and characterized. Next, the assay was used as a platform to screen a group of low-molecular-weight compounds, as well as black tea extract, for their ability to antagonize liposome permeabilization by Aβε peptide aggregates. Two different classes of small molecules were chosen, a group of 15 naturally-occurring polyphenols and another group of 8 synthetic N'-benzylidene-benzohydrazide (NBB) derivatives. Black tea extract provided a rich and complex milieu of bioactive polyphenols. Our conclusions enabled us to gain insight into particularly important structure-activity relationships which may have implications for development of disease-modifying therapy in AD.

**MATERIALS AND METHODS**

**Materials**

Purified synthetic Aβε was purchased from rPeptide (Bogart, GA, USA); phospholipids were purchased as a synthetic blend in chloroform: L,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG)/1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC).
Preparation of \( \alpha \beta_4 \) aggregates

Lyophilized \( \alpha \beta_4 \) was dissolved in 10 mM NaOH to 200 mM, and stored at \(-80^\circ\)C. The aggregation protocol was based on that of Necula et al. [49], which generates \( \alpha \beta \) oligomers and protofibrils. Thus, oligomerization was achieved by incubating 45 \( \mu \)M \( \alpha \beta_4 \) in sterile PBS (pH 7.4) for 2 h at 37 \( ^\circ \)C. To minimize evaporation, microfuge tubes (1.5 ml; Eppendorf, Germany) were sealed with Whatman\textsuperscript{\textregistered} adhesive film.

Thioflavin T fluorescence assay

Thioflavin T (ThT) is a dye widely used for the detection of cross-%beta-sheet structures in proteins, characteristic of the amyloid fibrillar form. The ThT assay was modified from El-Agnaf et al. [50]. Aggregation of \( \alpha \beta_4 \) was monitored over 24 h, at each sampling time, a 10 \( \mu \)l aliquot of the \( \alpha \beta_4 \) solution was diluted in 190 \( \mu \)l PBS (pH 7.5) containing 12 \( \mu \)M ThT. Fluorescence was measured in black 96-well plates using an FLx800-TBID microtiter plate reader (BioTek, Germany), with excitation at 440 nm and emission at 490 nm. To allow for background fluorescence, the fluorescence intensity of a blank PBS solution was subtracted from all readings. Data were measured in triplicates and averaged for evaluations.

Analysis of \( \alpha \beta_4 \) aggregation by immunoblotting

Immunoblotting of \( \alpha \beta_4 \) aggregation samples was performed using precast NuPAGE\textsuperscript{\textregistered} Novex4–12% Bis-Tris gels (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions. The samples were prepared in NuPAGE\textsuperscript{\textregistered} gel-loading buffer and resolved by subjecting to electrophoresis in 1x MOPS SDS buffer at 150 V for \(~1.5\) h. Samples were not boiled to avoid any oligomer dissociation. Transfer to nitrocellulose membrane (Hybond-ECL; General Electric, USA) was carried out using XCell II\textsuperscript{\textregistered} Blot Module (Invitrogen, Darmstadt, Germany), at 30 V for 1 h. After transfer, the membrane was immersed in 5% blocking reagent and left overnight at 4 \( ^\circ \)C. \( \alpha \beta_4 \) was detected using 6E10 monoclonal antibody (1 : 4000; Covance, Princeton, NJ, USA). Sites of primary antibody binding were detected with horseradish peroxidase-conjugated secondary antibody (1 : 5000) and blots were developed using ECLplus kit (General Electric, USA) according to the manufacturer’s instructions.

Preparation of Small Unilamellar Vesicles (SVs)

All vesicles used in our study were made up of the phospholipids DOPE:DOPS:DOPC (5 : 3 : 2). These vesicles have a net negative charge and lipid moieties native to the neuronal environment [51]. Typically, 50 \( \mu \)g of lipid was thoroughly dried from a chloroform solution under vacuum, to yield a thin lipid film at the bottom of a glass vial. The thin film was hydrated by adding 500 \( \mu \)l of a solution, 100 mM KCl, 10 mM MOPS/Tris, 1 mM EDTA (pH 7.0), containing 50 \( \mu \)g OG dye. The suspension was left to hydrate for 60 min, with vortexing approximately every 15 min until it turned very cloudy. Subsequently, 0.8 M Mega-9 detergent was added to the suspension, and it was vortexed gently until it appeared clear again. The lipid solution was then carefully loaded into SpectraPor\textsuperscript{\textregistered} (Spectrum Labs, Breda, The Netherlands) membrane tubing (pore size, 3.5 kDa) and subjected to dialysis for 6 h at room temperature against a buffer (5) containing 100 mM KCl, 10 mM MOPS/Tris (pH 7.0). This was followed by two successive overnight dialyses at 4 \( ^\circ \)C in the dark, with renewal of fresh buffer each time. Liposomes were retrieved from the tubing and kept at 4 \( ^\circ \)C covered in aluminum foil.

A similar protocol was used to prepare FITC-dextran loaded liposomes, but in this case the lipid film was resuspended in 500 \( \mu \)l of 2 mg/ml FITC-dextran (40 kDa, 70 kDa) whilst the untrapped FITC-dextran...
molecules were removed by 3–4 cycles of centrifugation (20,000 × g for 30 min). The size and uniformity of the vesicle population were checked using a Zetasizer Nano S dynamic light scattering (DLS) device (Malvern, Worcestershire, UK). The vesicles were relatively uniformly-sized with an average diameter of 67 ± 14 nm (data not shown), and hence categorized as SUVs.

Liposome permeabilization assays

Liposome permeabilization assays indicate disruption of lipid vesicle membrane by release of an encapsulated fluorophore [52]. In these experiments, the calcium-sensitive fluorophore Oregon Green and FITC-dextran molecules were used. In the case of OG, increased fluorescence resulting from inward calcium leakage and/or outward dye leakage indicates permeabilization of the liposomal membrane. In the case of FITC-dextran, FITC-dextran molecules entrapped in the liposomes are at high concentration and therefore self-quenching occurs; hence, leakage can be monitored as a relief of quenching. Fluorescence measurements were carried out using a microplate reader (FLx800-TBID; BioTek, Germany) set with excitation and emission wavelengths appropriate for the fluorophore being used (for OG: excitation = 485 nm, emission = 528 nm; for FITC: excitation = 495 nm, emission = 520 nm). Readings were taken in black 96-well microtiter plates (100 μl total well volume) at a constant temperature of 22°C. For kinetic readings, the reader was programmed to take measurements at regular intervals, typically for 15–20 min. Before each reading, shaking for 30 s ensured adequate mixing of the samples. Fluorescence intensity data was acquired according to the equation:

\[ \text{Permeabilization (\%)} = \frac{F - F_0}{F_{\text{max}} - F_0} \times 100 \]

where \( F \) denotes the maximum fluorescence intensity of the measured sample; \( F_{\text{max}} \) denotes the maximum fluorescence intensity after the addition of 2 μM ionomycin (a calcium ionophore) in the case of OG liposomes, or after the addition of 5% M-PER® (a membrane detergent) in the case of FITC-dextran liposomes; and \( F_0 \) represents the fluorescence of intact vesicles. Fluorescence intensity values are given in RFU.

Assays for inhibition of Aβ42-induced liposome permeabilization

1 μM aggregated Aβ42 was incubated for 10 min with compounds in assay buffer, prior to the addition of 25 μM liposomes. Disruption of lipid vesicles by Aβ in the presence of compound was calculated as a percentage of permeabilization caused by Aβ alone (theoretical maximum, 100%). Tested compounds included: (i) 15 natural plant polyphenols (typically at 50 μM): Api, Bac, Cat, EGCG, Fos, Gen, Gink, Mor, Myr, NDGA, Purp, Que, Roru, Resv and Scut; (ii) BTE (typically at 15 μg/ml); (iii) 8 NBB derivatives (typically at 10 μM). At these typical concentrations none of the compounds had any significant destabilizing effect on the liposomal membranes. Autofluorescence (where necessary) was measured for each compound and was subtracted from the sample well values.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc.). Results were expressed as the means and the standard error of the mean (SEM) values, with n as the number of experiments. Differences between means were determined using unpaired Student’s t test. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Characterization of Aβ42 aggregates

In a working hypothesis of AD, Aβ aggregates (typically, oligomers) directly damage neuronal and synaptic membranes, leading to ionic dyshomeostasis and eventually neurodegeneration. We therefore wanted to generate Aβ42 multimeric species, mainly oligomers, that robustly compromised membrane permeability of SUVs. The latter vesicles are a representative model of native brain neuronal membranes, having a net negative charge and similar phospholipid composition.

The kinetics of Aβ42 aggregation were first monitored by measuring ThT fluorescence [53]. As expected, there was a lag phase (with no change in cross-β-sheet structure) during the first 2 h of incubation, followed by a 4-h growth phase during which Aβ42 peptides aggregated into an increasingly fibrillar structure (Fig. 1A). Such fibrilization kinetics are consistent with a nucleated polymerization mechanism and hence
Aβ42 oligomers evoke dose-dependent permeabilization of DOPE:DOPS:DOPC liposomes

A permeabilization assay was conducted, comparing the perforation ability of Aβ42 aggregates formed after 1, 2, or 3 h incubation at 37°C. The strongest permeabilization effect was observed with the 2-h and 3-h aggregates (31.2% and 30%, respectively; Fig. 2), whilst Aβ42 peptides incubated for only 1 h had a significantly weaker effect (23.9%; p = 0.027). Therefore, on the basis of a combination of low ThT fluorescence and robust membrane perforation, we opted for the 2 h aggregation to standardize our Aβ oligomerization protocol. Moreover, Aβ42 multimers resulting from a 2 h aggregation were separated and visualized by immunoblotting (Fig. 1B). This confirmed formation of oligomers ranging in size from 45–220 kDa, i.e., in the high-molecular-weight (HMW) range. These oligomers were capable of inducing immediate permeabilization of SUVs at 0.25–5 μM (in moles of monomeric peptide), in most cases reaching a maximal effect within 15 min (Fig. 3). Permeabilization by the Aβ oligomers was calculated as percentage of the ionomycin effect (a calcium ionophore): 15.7% ± 1.3 (0.25 μM), 24.7% ± 7.5 (0.5 μM), 25.8% ± 5.6.
measurements yielding quantitatively identical results. Were obtained from one experiment, out of at least three independent 
vesicle permeabilization by Aβ oligomers (Fig. 3). These results suggest that 0.5–1 μM aggregated Aβ2 was sufficient to cause defects in the lipid bilayer that result in increased membrane permeability. However, a 1 μM concentration was more robust than 0.5 μM in giving a minimum permeabilization of more than 20% (0.5 μM: 24.7% ± 7.5; 1.0 μM: 25.8% ± 5.6). Therefore, 1 μM Aβ2 was typically chosen for subsequent experiments.

Finally, we wanted to characterize better the mode of membrane destabilization by Aβ, for instance, whether by channel, pore formation or total disruption of the vesicle membrane. Thus, a size-dependent fluorescence leakage assay was performed by preparing liposomes containing FITC-dextrans of two different molecular sizes (40 kDa or 70 kDa) and exposing them to 1 μM Aβ2 oligomers. The leakage of the differentially-sized markers occurred as follows: 46.9% ± 2.6 (1 kDa), 38.3% ± 2.6 (40 kDa), and 39.3% ± 2.9 (70 kDa). Although there was a minor decrease in leakage of the larger-sized dextrans, this difference was not statistically significant (p = 0.07).

**Inhibitory effects of natural polyphenols and BTE on lipid vesicle permeabilization by Aβ2 oligomers**

Having established a reproducible protocol for lipid vesicle permeabilization by Aβ2 oligomers, we proceeded to test the inhibitory effects of 15 plant polyphenols and BTE. The compounds were allowed to interact with pre-aggregated Aβ2 before adding liposomes; this approach allowed the compounds to antagonize vesicle disruption by destabilizing the toxic Aβ species and/or by interfering with the binding of Aβ oligomers to the lipid vesicle membranes. The results are summarized in Fig. 4A. Overall, addition of plant polyphenols and BTE led to a decrease in the vesicle permeabilization induced by Aβ2 aggregates alone, ranging from 90% permeabilization (weak inhibitory effect) to 4% permeabilization (very strong inhibitory effect). Compounds were hence classified as follows:

- **Group I** - very strong inhibitors (<10% permeabilization) BTE (4%).
- **Group II** - strong-to-moderate inhibitors (10–49% permeabilization) Baic (22%), Apis (27%), NDGA (29%), Purp (35%) and Scut (45%).
- **Group III** - moderate-to-weak inhibitors (50–79% permeabilization) Rosm (58%), EGCG (67%) and Quer (73%).
- **Group IV** - weak inhibitors (80–95% permeabilization) Myr (80%), Cat (80%), Gen (80%), Mor (81%), Fis (82%), Gink (84%) and Resv (90%).

A select group of three compounds from Groups I and II (BTE, NDGA and Purp) were subsequently tested for concentration-dependency in the range 10–100 μM (or 1–15 μg/ml in the case of BTE), using the liposome permeabilization assay. NDGA remained moderately effective even at 20 μM (60%), whilst BTE was still a potent inhibitor at 5 μg/ml (<10%; Fig. 4B).

**Synergistic inhibition by pairwise combinations of polyphenols**

It was hypothesized that combinations of small-molecule compounds could manifest a stronger, synergistic, inhibitory effect than either compound alone. Thus, pairwise associations of Api, NDGA, and Purp were investigated for their inhibition of Aβ-induced lipid vesicle membrane destabilization (Fig. 5). A synergistic effect was observed only with the Purp-API combination: 10 μM Purp alone (94%), 10 μM Apis alone (80%), 10 μM Purp-Apis (48%). The other combinations showed at most additive, but not synergistic, effects (data not shown).
**Fig. 4.** Effects of polyphenols and black tea extract on lipid vesicle permeabilization by Aβ42 oligomers. A) Polyphenols (50 μM) and BTE (15 μg/ml) were incubated for 10 min with 1 μM pre-aggregated Aβ42, after which 25 μM liposomes were added to monitor permeabilization. Based on their inhibitory effects, the compounds were classified into 4 groups: very strong (black column), strong-to-moderate (dark gray columns), moderate-to-weak (light gray columns), and weak (white columns). B) Concentration-dependent inhibition of liposome permeabilization by purpurogallin trimethyl ether (Purp), NDGA and black tea extract (BTE). Liposome permeabilization in presence of compounds is given as a percentage of the effect of Aβ alone (% Aβ42, y-axis). Values represent means ± SEM (n ≥ 3); *p < 0.05, **p < 0.01, ***p < 0.005 versus Aβ42.

**Inhibition of Aβ42-induced lipid vesicle permeabilization by synthetic N′-Benzylidene-Benzohydrazide (NBB) compounds**

Next, eight synthetic NBB compounds (NBB #1–8) were also tested by the liposome permeabilization inhibition assay (at 10 μM). The NBB compounds were compared directly to the top natural polyphenols identified by the previous screening (also at 10 μM). This allowed identification of three NBB compounds that were as potent as Baic, Api, and NDGA in protecting lipid vesicle membranes from Aβ permeabilization (Fig. 6): NBB #6 (80%), NBB #7 (80%) and NBB #8 (67%).
DISCUSSION

Maintenance of plasma membrane integrity is crucial for neuronal function. In this regard, there is accumulating evidence that prefibrillar Aβ oligomers are the primary neurotoxic species in AD [55] and that disturbance of neuronal membranes is a likely first step in the pathophysiological cascade initiated by these oligomers [56]. Therefore, we first sought to generate soluble high-molecular-weight Aβ oligomers and confirmed that they robustly compromised membranes of model unilamellar liposomes at near-physiological concentrations. Of note, ‘fresh’ Aβ peptides (consisting of monomers and very small oligomeric species) were unable to induce vesicle permeabilization. This is in agreement with several reports of increased membrane conductance and permeability by prefibrillar Aβ oligomers, but not by near-monomeric species [17, 57–59]. Large assemblies of Aβ molecules have in fact been detected attached to live cell membranes [60]. As a point of clarification, it cannot be excluded that further conformational conversion takes place upon interaction of the oligomers with lipid membranes. For example, phosphatidylcholine...
the conversion of attached prefibrillar oligomers into pore-like annular protofibrils [61].

Most of the fluorescent dye leakage occurred rapidly during the first 5 min, similar to what has been described previously [62]. In order to explore the possibility that the Aβi aggregates might be forming pores within liposome membranes, the differences in leakage between 1-kDa, 40-kDa, and 70-kDa fluorophores was determined and found not to be statistically significant, implying either complete disruption of the membrane or a large-pore forming mechanism. Interestingly, it has been reported that pores formed by Aβi in situ, in the neuronal cell membrane in AD brain, had a lumen of ∼10 nm [63]. Such large Aβi pores would be expected to allow molecules, certainly up to the size of 100 kDa, to leak out freely. Therefore, a permeabilization mechanism involving pore formation by the HMW Aβ42 aggregates cannot be excluded a priori. It is relevant to point out here that pore-forming proteins, like the bacterial toxin α-hemolysin and human perforin, share structural homology with amyloid oligomers suggesting a common mechanism of membrane permeabilization [64].

The ability of small molecule compounds and black tea extract to protect phospholipid membranes from disruption by Aβi aggregates was tested using our permeabilization assay. Out of 15 natural polyphenols, 8 N-benzylidene-benzohydrazide derivatives and BTE, the most active was in fact the tea extract. At 5 μg/ml, BTE still prevented the membrane-disordering effect of Aβ42 oligomers to less than 10% of control Aβi alone. Our assay result is supported by recent human epidemiological and animal data suggesting that tea drinking may help protect the aging brain and reduce the incidence of dementia or AD [65]. Thus, interfering with oligomer-induced lipid membrane damage may represent a key modality in the treatment of AD neurodegeneration.

The basic flavonoid structure is the flavan core, which consists of a 15 carbon skeleton (C6-C3-C6), arranged in three rings labeled as A, B, and C (Fig. 7). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring. These include the flavonols (e.g., Fis, Mor, Myr, Quer), the flavanols (e.g., EGCG, Cat), the flavones (e.g., Api, Baic, Scut), and the isoflavones (e.g., Gen). Individual compounds within a class differ in the pattern of substitution of the A and B rings. Apart from the very strong activity of BTE, five small-molecule polyphenols were classified as “strong-to-moderate” inhibitors since they decreased liposome permeabilization to 10–49%. Notably, of these five compounds, three are flavones: 5,6,7-trihydroxyflavone (Baic), 4′,5,7-trihydroxyflavone (Api), and 4′,5,6,7-tetrahydroxyflavone (Scut). Examining these three structures, a common 5,7-dihydroxyflavone scaffold appears to be a prominent feature (Fig. 7). In contrast, the weaker flavonols Fis, Mor, Myr, and Quer possess a hydroxyl group attached to C3. Moreover, Cat and EGCG, as well as the isoflavone Gen, have the B ring of the flavonoid molecule attached to C3. Evidently, substitutions at the C3 position have profound negative effects on the ability of the polyphenol molecule to attenuate membrane permeabilization.

Another strong inhibitor of lipid membrane damage by the Aβ42 oligomers is NDGA, which shares a 6,7-dihydroxyphenyl ring structure with the A ring of Baic and Api (Fig. 7). Interestingly, dihydroxyphenyl rings are a recurring feature of the three synthetic NBB compounds (NBB #6–8, 307F06, 301C09, and 293G02, respectively) with inhibitory activities similar to Api, Baic, and NDGA. To conclude, intuitive recognition of the structural relationships associated with highest inhibitory activity allows derivation of the ‘scaffold tree’ shown in Fig. 7: essentially, a dihydroxyphenyl ring alone, or as part of a flavone module with a preferred maximum of 3 hydroxyl groups, is particularly adapted for protection against membrane damage by Aβi oligomers. These structure-activity relationships may also be of importance when considering treatment with combinations of small-molecule compounds. For instance, we noted a synergistic effect between Api and Purp: the paired compounds at 10 μM were almost as strong as either compound alone at five times that concentration. The synergism implies a different mode of action of these two molecules, which represent in fact a combination of an apolar (Purp) and polar (Api) chemical structures.

The molecular mechanisms underlying preservation of membrane integrity by BTE and photonic compounds is currently under investigation. First of all, rescue may depend upon interaction of compounds with the oligomers themselves. This would lead to dissociation and/or remodeling of the oligomers into harmless Aβ species. Substantial data has been published on the ability of the studied inhibitors to inhibit Aβ aggregation and/or destabilize formed assemblies in vitro [66–69]. Importantly, however, in all of these assays Aβi aggregation was tested in the absence of lipids. Indeed, when comparing these reported anti-amyloidogenic properties of polyphenols, on one hand, with our results from the liposome
Fig. 7. Chemical structures of the top small-molecule compounds inhibiting Aβ-induced liposome permeabilization. The flavone polyphenols: apigenin (4′,5,7-trihydroxyflavone), baicalein (5,6,7-trihydroxyflavone) and scutellarein (4′,5,6,7-tetrahydroxyflavone). A shared criterion is thus the 5,7-dihydroxyflavone structure; note the 15 carbon skeleton (C6-C3-C6) of a flavan core, arranged in three rings labeled as A, B, and C.

Another strong inhibitor was found to be the polyphenol NDGA, which shares a 6,7-dihydroxyphenyl ring structure with the A ring of baicalein and apigenin. Dihydroxyphenyl rings are common to the three top-scoring N′-benzylidene-benzohydrazide compounds (307F06, 301C09 and 293G02).

permeabilization assay, on the other, we find no significant correlation between the two. For example, Api was found to be a very weak inhibitor of Aβ aggregation (IC50 > 40 μM), and Baic (IC50 = 5.3 μM) was at least an order of magnitude less potent than other flavonoids such as Myr (IC50 = 0.67 μM), Mor (IC50 = 0.4 μM), Quer (IC50 = 0.72 μM), or EGCG (IC50 = 0.18 μM). These observations seem to suggest, therefore, that mechanisms involving direct binding of the compounds to Aβ multimeric structures leading to their complete disassembly [31] are not likely to be primarily involved. The possibility still remains, notwithstanding, that compounds like Api and Baic remodel the soluble oligomers into larger aggregates that are non-toxic to membranes [70]. The exception appears to be NDGA, which has a known potent anti-amyloidogenic and fibril destabilizing activity (IC50 = 0.74–1.1 μM) [69]. NDGA is composed differently from the flavones, and has two relatively polar aromatic groups (the dihydroxyphenyl rings discussed...
above) connected by a rigid linker. Interestingly, effective molecules from the NBB derivates (NBB #6–8) have an analogous structure to NDGA, with a dihydroxyphenyl ring at one end of the molecule, a phenyl ring at the other and a central linker. It is also striking that the top NBB compound in our liposome permeabilization assay, NBB #8 (293G02), correlates nicely with previously published findings. 293G02 was also the best NBB compound to demonstrate anti-prion activity in a high-throughput screening assay [71], to potently inhibit polyQ aggregation [72] and to interfere with alpha-synuclein oligomer formation in living cells [73].

Another possible mechanism would involve modulation of the oligomer-membrane interaction by the compounds, particularly the polyphenols. A flavonoid-dependent effect on the lipid bilayer may explain, in part, the preservation of membrane integrity by the compounds. It has been shown recently that flavonoids partially penetrate bilayer membranes, resulting in a strong rigidifying effect on the bilayer structure. Consequently, membranes exposed to flavonoids loose the ability to interact with Aβ peptide [40].

CONCLUSION

We report here a novel study on the effects of small-molecule natural and synthetic compounds, and black tea extract, on the permeabilization of lipid vesicles by Aβ42 oligomers. Based upon our data, we encourage more in-depth studies on the potential disease-modifying effects of black tea in AD. Apart from tea extract, the common structural criteria of the NBB derivates (NBB #6–8) connected by a rigid linker. Interestingly, effective molecules from the NBB derivates (NBB #6–8) have an analogous structure to NDGA, with a dihydroxyphenyl ring at one end of the molecule, a phenyl ring at the other and a central linker. It is also striking that the top NBB compound in our liposome permeabilization assay, NBB #8 (293G02), correlates nicely with previously published findings. 293G02 was also the best NBB compound to demonstrate anti-prion activity in a high-throughput screening assay [71], to potently inhibit polyQ aggregation [72] and to interfere with alpha-synuclein oligomer formation in living cells [73].

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