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Modulating Self-Assembly of Amyloidogenic Proteins as a Therapeutic Approach for Neurodegenerative Diseases: Strategies and Mechanisms

Tingyu Liu[b] and Gal Bitan*[a]

Abnormal protein assembly causes multiple devastating disorders in the central nervous system (CNS), such as Alzheimer's, Parkinson's, Huntington's, and prion diseases. Due to the now extended human lifespan, these diseases have been increasing in prevalence, resulting in major public health problems and the associated financial difficulties worldwide. The wayward proteins that lead to disease self-associate into neurotoxic oligomers and go on to form fibrillar polymers through multiple pathways. Thus, a range of possible targets for pharmacotherapeutic intervention exists along these pathways. Many compounds have shown different levels of effectiveness in inhibiting aberrant self-assembly, dissociating existing aggregates, protecting cells against neurotoxic insults, and in some cases ameliorating disease symptoms in vivo, yet achieving efficient, disease-modifying therapy in humans remains a major unattained goal. To a large degree, this is because the mechanisms of action for these drugs are essentially unknown. For successful design of new effective drugs, it is crucial to elucidate the mechanistic details of their action, including the actual target(s) along the protein aggregation pathways, how the compounds modulate these pathways, and their effect at the cellular, tissue, organ, and organism level. Here, the current knowledge of major mechanisms by which some of the more extensively explored drug candidates work are discussed. In particular, we focus on three prominent strategies: 1) stabilizing the native fold of amyloidogenic proteins, 2) accelerating the aggregation pathways towards the fibrillar endpoint thereby reducing accumulation of toxic oligomers, and 3) modulating the assembly process towards nontoxic oligomers/aggregates. The merit of each strategy is assessed, and the key points to consider when analyzing the efficacy of possible drug candidates and their mechanism of action are discussed.

Introduction

Recent studies have suggested that there is a common culprit—toxic, aberrantly folded and aggregated proteins—in over 30 human disorders that currently have no cure. These disorders can affect the central nervous system (CNS), as in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and familial amyotrophic lateral sclerosis (fALS), other organs, such as the pancreas in type II diabetes or the heart in familial amyloidotic cardiomyopathy (FAC), or the entire body, as in systemic amyloidoses.[1] In each disorder, a particular protein (or proteins) is implicated as causative: Aβ and hyperphosphorylated tau (p-tau) in AD,[2] α-synuclein (α-syn) in PD,[3] mutant huntingtin (Htt) with abnormally extended polyglutamine (polyQ) repeats in HD,[4] mutant superoxide dismutase-1 (SOD-1) in fALS, islet amyloid polypeptide (IAPP) in type II diabetes,[5] and transthyretin (TTR) in FAC.[6]

Many therapeutic strategies targeting the origin and/or toxic effect of amyloidogenic proteins have been pursued to prevent or cure the associated diseases. A comphrehensive overview of these strategies can be found in a review written by Bartolini and Andrisano.[7] One prominent strategy is reducing the production of the offending protein. For example, in AD, inhibitors of β- or γ-secretases, the enzymes that release Aβ from the amyloid β-protein precursor (APP), have been developed by multiple academic laboratories and pharmaceutical companies.[8] Another strategy is promoting clearance of the offending proteins, for example, by immunotherapy,[9] activation of specific proteases,[10] or by general clearance mechanisms.[11] Additional strategies attempt to modulate the detrimental effects of the disease indirectly rather than to target the offending proteins. For example, attempts have been made to inhibit mitochondrial dysfunction, which is often related to oxidative stress and impaired ATP production.[12] Using general protective and neurotrophic agents is another commonly used strategy. Neurotrophic factors, such as neurotrophin, brain-derived neurotrophic factor, activity-dependent neuroprotective protein, and others,[13] might protect against cell death and promote neurogenesis to reduce the synaptic and cognitive damage associated with some of these diseases.[14]

In this Minireview, we focus on therapeutic strategies that directly target the aggregation process of amyloidogenic proteins. Much of the research on such strategies has focused on

[a] *Dr. G. Bitan*
Department of Neurology, David Geffen School of Medicine, and
Brain Research Institute and Molecular Biology Institute
University of California, Los Angeles
635 Charles E. Young Drive South/NRB 455, Los Angeles, CA 90095 (USA)
E-mail: gbitan@mednet.ucla.edu

[b] *T. Liu*
Department of Neurology, David Geffen School of Medicine
University of California, Los Angeles
635 Charles E. Young Drive South/NRB 455, Los Angeles, CA 90095 (USA)

Aβ, which is often considered an archetypal amyloid protein. Nonetheless, abundant evidence demonstrates that although other amyloidogenic proteins share little sequence similarity with Aβ, the structures involved in the aberrant assembly process, including various oligomers, protofibrils, and fibrils, are shared by virtually all other amyloidogenic proteins.

Amyloidogenic proteins can be divided into two classes. The first class comprises natively structured proteins that often undergo amyloidogenic transformation due to mutations that alter their structures, or cause overproduction or deficient clearance. TTR, SOD-1, and possibly α-syn belong to this group. Proteins in the second class are natively unstructured and include, for example, Aβ, tau, and IAPP. In addition to genetic causes, proteins in both classes can go down the amyloid pathway due to changes in the environment or post-translational modifications in the absence of mutations. The pathogenic transformation begins when the protein, whether structured or unstructured, forms partially (un)folded intermediates, in which aggregation-prone sequences are exposed and bind to each other in an unnatural form leading to disease (Figure 1).

Following the initial association of two monomers into a dimer and before formation of amyloid fibrils, the assemblies are classified loosely as oligomers—an imprecise definition describing a variety of species that are water-soluble, metastable, inevitably exist as mixtures of multiple structures, and in most cases are cytotoxic.[15] Within the broad definition of oligomers, the penultimate species, which is common to most amyloid formation processes,[16] is the protofibril, which was first defined in 1997 by the Teplow[17] and Lansbury[18] groups, independently. Protofibrils are fibril-like structures that, unlike mature fibrils, are metastable, curvilinear, relatively short (100–200 nm) and narrow (~5 nm in diameter). Protofibrils can disassemble back into oligomers,[17] but once protofibrils mature into fibrils, the latter are insoluble and cannot easily disassemble.[19] Multiple studies have demonstrated that oligomers are

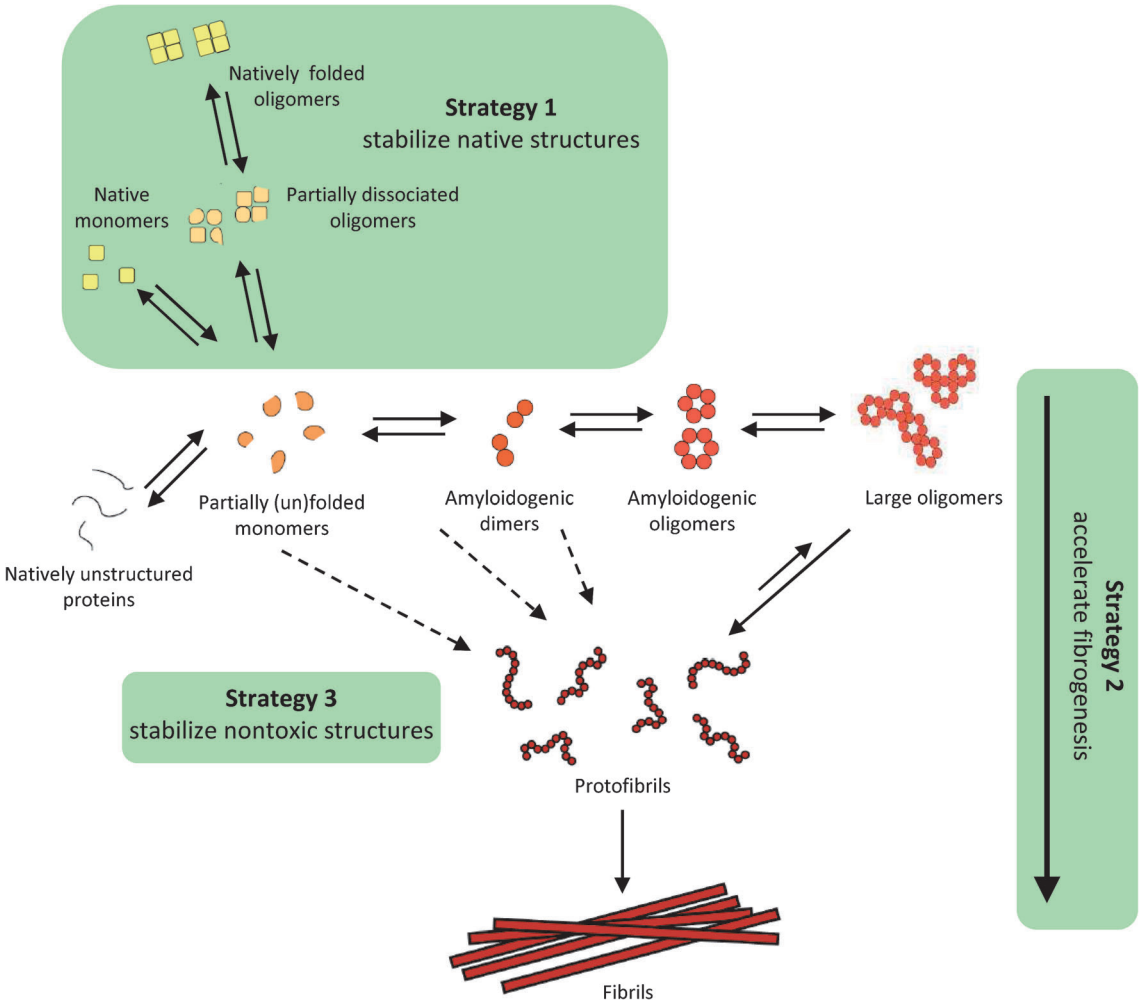


Figure 1. Schematic representation of the aggregation pathway of amyloidogenic proteins and the three therapeutic strategies covered in this Minireview. Natively folded oligomers, monomers, and unstructured proteins can partially fold/unfold into monomers that harbor amyloidogenic characteristics, leading them to aggregate into increasingly larger, toxic oligomers. These structures assemble via multiple, simultaneous pathways and ultimately transform into fibrils. Strategy 1 stabilizes the native protein structures and prevents unfolding. Strategy 2 accelerates fibril formation and decreases steady-state oligomer concentration. Strategy 3 modulates the assembly process by stabilizing nontoxic structures that can form upon interaction of the compounds with monomers, oligomers, and/or fibrils.

more toxic than their counterpart fibrils and that fibrils might even harbor a protective role. This hypothesis is crucial when considering therapeutic targets for drug development that inhibit or modulate the aberrant assembly process.[20]

It is important to note that different aggregation pathways have been proposed and most likely, multiple pathways exist simultaneously, both in vitro and in vivo.[21] The coexistence of pathways in which self-assembly either precedes or follows conformational transition has been demonstrated in an elegant study using the model protein barstar.[22] The nucleation-dependent polymerization model[23] suggests a relatively simple picture, according to which monomers first self-associate into nuclei or "seeds" in a slow, rate-determining step, followed by rapid elongation of fibrils in which monomers are added one at a time. According to this model, the growing tips of amyloid fibrils serve as templates onto which monomers first dock to form relatively loose structures, and then "lock" into place by additional conformational changes leading to substantial structure stabilization.[24] A different pathway involves self-association of monomers into small oligomers, which then self-assemble to form larger oligomers, and protofibrils (Figure 1).[25]

The multiple parallel assembly pathways and the simultaneous existence of various metastable structures pose tremendous challenges in tackling amyloid-related diseases. Are particular stages in the aggregation pathways the most pivotal? Are specific species the most detrimental? Can we even distinguish reliably between the different stages and species? For therapies based on inhibition or modulation of protein aggregation to be effective, it is necessary to elucidate which elements of the aggregation pathways drug candidates are targeting, how and what they bind to, and what mechanisms they work by, both in vitro and in vivo. Although many drug candidates have been reported to inhibit aggregation, their mechanisms of action remain largely unclear. Understanding these mechanisms will allow educated design and development of compounds in an effective and efficient manner. Here, we discuss promising drug candidates targeting aberrant protein assembly with particular attention to the mechanisms by which they work. To lead the discussion, we group these mechanisms into three broad strategies, each targeting different aspects of the self-assembly pathway (Figure 1).

Strategy 1: Stabilizing the Monomer

Perhaps the most obvious strategy is to prevent the very first step of the aggregation process, in which a protein partially unfolds. Theoretically, this can be achieved using compounds that stabilize the native protein structure and prevent it from unfolding. Naturally, this strategy is possible only for proteins that have a stable structure.

The most advanced research along this vein is on TTR, a plasma protein whose aberrant aggregation causes several disorders, such as FAC, familial amyloid polyneuropathy (FAP), and senile systemic amyloidosis (SSA). The native form of TTR is a homotetramer (Figure 2a). Its main physiologic role is to transport retinol and thyroxine in the plasma. Over a hundred mutations in the *TTR* cognate gene have been reported to produce amino acid substitutions that decrease the thermodynamic and/or kinetic stability of TTR, causing partial unfolding of the tetramer leading to formation of toxic, amyloidogenic structures.[26] The TTR tetramer contains two binding sites for thyroxine, which are frequently unoccupied.[27] Several benzoxazoles[28] and diflunisals[29] (Figure 2b) have been reported to act as kinetic stabilizers of TTR by binding to the unoccupied thyroxine binding sites, thereby increasing the activation energy necessary for the protein to unfold.[30] In particular, tafamidis meglumine (Figure 2c) has completed an 18-month phase II/III clinical trial (Fx-005),[31] and based on the promising results, this agent continued on to an open-label, 12-month extension study (Fx-006). Results from both studies revealed that patients treated with the drug had less deterioration of neurological function, as determined by the Neuropathy Impairment Score–Lower Limb (NIS–LL) parameter, compared with patients treated with placebo. In November 2011, the European Commission approved the use of the drug for the treatment of TTR–FAP.[32]

Stabilization of the native form has recently been applied to SOD-1, an enzyme that exists as a native homodimer. As with

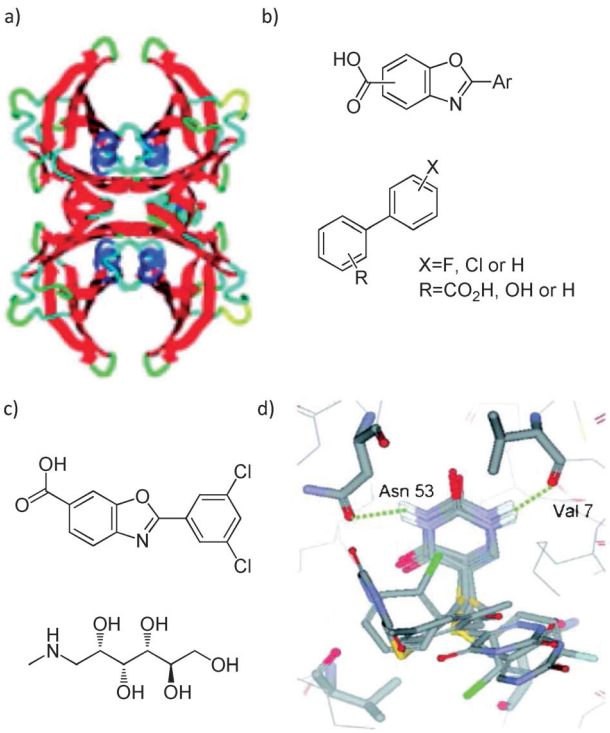


Figure 2. Stabilization of the native form to prevent amyloidogenic transformation. a) Ribbon representation of the X-ray crystallographic structure of homotetrameric wild-type transthyretin (TTR). (Adapted with permission from C. J. Simões, T. Mukherjee, R. M. Birto, R. M. Jackson, *J. Chem. Inf. Model.* **2010**, *50*, 1806–1820. Copyright 2010 American Chemical Society). b) Benzoxazoles (top) and diflunisals (bottom): two classes of TTR kinetic stabilizers. c) Tafamidis meglumine, a drug approved in the EU for the treatment of familial amyloid polyneuropathy (FAP). d) Docked small-molecule stabilizers occupying the binding site on the dimer-interface region of superoxide dismutase-1 (SOD-1) and forming hydrogen bonds with Asn53 and Val7. (Adapted with permission from R. J. Nowak, G. D. Cuny, S. Choi, P. T. Lansbury, S. S. Ray, *J. Med. Chem.* **2010**, *53*, 2709–2718. Copyright 2010 American Chemical Society).

TTR, there are over a hundred mutations in the gene encoding SOD-1 that lead to fALS, a fatal motor neuron disease. A recent study has found a class of small molecules that stabilize the familial mutant SOD-1 A4V by binding in a pocket at the dimer interface (Figure 2 d). These molecules were found by docking simulations using a library of approximately 2.2 million compounds with four hydrogen-bond constraints and then assessing the top hits for their ability to block aggregation of SOD-1 A4V using size-exclusion chromatography (SEC).[33] It will be interesting to see future studies examining the ability of these compounds to reduce toxicity in cell and animal models of fALS.

α-Syn is a protein for which aberrant aggregation is implicated as causative in PD.[34] Until recently, it was largely regarded as a natively unstructured monomeric protein that assumed a predominantly α-helical conformation upon association with membranes.[35] However, recent studies have proposed that α-syn might exist endogenously as a folded tetramer, which was not previously detected due to denaturation during its purification from recombinant sources.[36] If indeed α-syn is a native tetramer, stabilization of its native structure using small molecules, similar to the effect of tafamidis meglumine on TTR, could be a viable therapeutic strategy for the prevention and treatment of PD and other diseases caused by α-syn aggregation (synucleinopathies).

In contrast to the examples listed above, for naturally unstructured proteins, the lack of a stable folded conformation makes it difficult to obtain high-resolution structures using solution-state nuclear magnetic resonance (NMR) or X-ray crystallography,[15b,37] which are necessary for computer-aided screening of drug candidates, as was done for SOD-1 A4V. Thus, stabilization of the native structure cannot be used for naturally unstructured amyloidogenic proteins.

Strategy 2: Accelerating the Pathway

Following the discovery that mature amyloid fibrils are less toxic than soluble oligomers, acceleration of the transition from oligomers to fibrils has emerged as a potential therapeutic strategy. This strategy might seem counterintuitive in light of the original amyloid cascade hypothesis,[38] which proposed that fibrils are the toxic species causing disease. However, multiple studies with different amyloidogenic proteins have demonstrated that the soluble and/or oligomeric form of the protein correlates better with disease severity than protein deposition.[39] For example, studies of mutant Htt have suggested that neuronal deposition of fibrillar aggregates resulted in improved neuronal survival and decreased levels of Htt elsewhere in the neuron.[40] A similar phenomenon has been observed with aggregated deposits of α-syn, termed Lewy bodies, which are a hallmark of PD.[3] Formation of Lewy bodies is poorly associated with cell toxicity and has been proposed to have a protective role, although the latter hypothesis is controversial.[41] If fibrils are less toxic than oligomers and protofibrils (for a review, see Ref. [42]), accelerating the aggregation would decrease the steady-state concentration of oligomers and could protect against the associated cytotoxic insults.

Methylene blue

Methylene blue (MB; Figure 3) is a widely studied compound that, at least in some cases, has been shown to accelerate fibril formation. In addition, this phenothiazine derivative effectively decreases oxidative stress and inflammation in the brain (for a review, see Ref. [43]). MB has many of the qualities of a drug candidate for neurodegenerative diseases—high aqueous solubility, low toxicity, and penetration across the blood–brain barrier (BBB).[44]

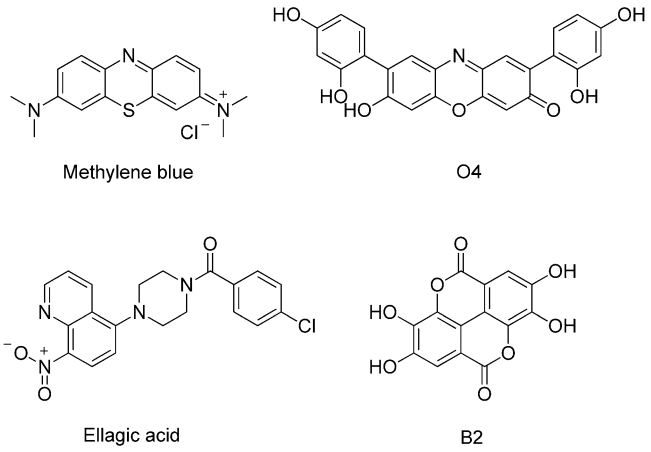


Figure 3. Drug candidates whose proposed mechanism of action is the acceleration of fibril formation.

The effect of MB on Aβ in vitro has been examined by Necula et al., who used dot blots with oligomer-specific antibody A11[45] coupled with transmission electron microscopy (TEM) to reveal that MB enhanced Aβ42 fibrillization leading to reduced oligomer accumulation.[46] In contrast to its effect on Aβ, MB appears to prevent tau self-association. An early study by Wischik et al. demonstrated that the compound disrupted tau self-association and decreased the stability of neurofibrillary tangles (NFTs), one of the two hallmarks of AD.[47] A study by Taniguchi et al. showed that MB inhibited heparin-induced tau filament formation. These studies suggest that MB has opposing effects on Aβ and tau. However, in the study by Taniguchi and co-workers, ultracentrifugation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that incubation with MB resulted in a decrease of monomeric tau concentration and the appearance of high molecular weight (HMW) tau bands, suggesting that the compound induces tau aggregation.[48] These data should be interpreted cautiously because SDS-PAGE can induce artifactual aggregation and disaggregation of amyloidogenic proteins.[15c] Additionally, cellular measures of toxicity used in conjunction with biophysical characterizations are necessary to delineate the relationship between effect of MB on protein aggregation and its impact on cell viability.

MB has been used for over a hundred years to treat illnesses ranging from schizophrenia to urinary tract infections. Thus, the efficacy and safety of MB have been relatively well charac-

terized.[49] An early study of the compound by Wischik led him to found TauRx Therapeutics, which has performed phase II trials with a proprietary formulation of MB (Rember). After receiving this drug in a gel capsule form for one year, dementia progression was slowed by 81% in AD patients compared to placebo, as assessed by the clinical test, Alzheimer's disease assessment scale–cognitive (ADAS-cog).[50] Researchers at TauRx Therapeutics are working to further improve and refine the formulation before progressing to phase III trials, especially as they have found that at higher doses, the compound loses the cognitive benefit and causes adverse side effects, such as gastrointestinal problems.[51]

Rember is marketed as a tau aggregation inhibitor, and TauRx Therapeutics has performed studies with transgenic mice that overexpress a gene encoding part of the human tau protein (amino acids 295–390) to further elucidate its mechanism.[52] When these mice were treated intravenously for 17 days (2 or 5 mg kg^{-1} day^{-1}), they showed reduced reactivity with an antibody that reacts with a human tau epitope buried within paired helical filaments (PHF), which becomes exposed following formic acid treatment[53] in the hippocampus and entorhinal cortex.[54] In a separate study, transgenic mice treated orally for two weeks (15 or 45 mg kg^{-1} day^{-1}) were subjected to a modified water maze protocol[55] and showed improvement in spatial memory deficits.[56]

Following the promising results from the clinical trial conducted by TauRx Therapeutics, other investigators conducted animal studies with unmodified MB and found conflicting results. Van Bebber et al.[58] assessed the effect of the compound in a zebrafish model with transgenic neuronal expression of the familial frontotemporal dementia (FTD)-associated tau–P301L mutation.[57] Zebrafish embryos were incubated with MB (100 μM in buffer) from 21 h to six days. The compound had no effect on the pathological conditions induced by the mutant tau, which include abnormal phosophorylation, tau-dependent neurotoxicity, and reduced escape response due to impaired axonal outgrowth of motor neurons, contradicting the results of the Rember studies. However, it is difficult to compare these results directly due to the difference in compound formulation, animal model, experimental design and set-up. In particular, the studies conducted by Wischik suggested that the preparation and formulation of MB were highly important for its effectiveness. The MB used in the zebrafish study was purchased from Sigma with 90% purity, whereas the material used by TauRx Therapeutics was a proprietary formulation with over 99% purity. In addition, low amounts of sarkosyl-insoluble tau in the transgenic zebrafish model did not allow for rigorous analysis of tau aggregation and deposition.

Van Bebber et al. also assessed the effect of MB on zebrafish injected with poly-Q-expanded fragments of Htt.[58] A filter trap analysis suggested that MB reduced aggregation of these poly-Q-containing fragments in a dose-dependent manner although there were no changes in toxicity. This result further complicates the assessment of the effects of MB, though it is consistent with studies suggesting that poly-Q fibril formation is not necessarily associated with toxicity.[40,59]

In a separate study, O'Leary et al. administered MB orally ad libitum (10 mg kg^{-1} in drinking water) to seven-month-old transgenic mice expressing the P301L mutation in human tau (rTg4510)[39c] for 12 weeks. The mice displayed moderate improvement in spatial memory (measured by the Morris water maze, MWM) and no changes in immunohistochemical (IHC) analysis of a p-tau epitope (S202/T205), MC1 epitope (early tangles), or the gallyas silver-stain (late tangles). There was, however, significant reduction in soluble tau concentration levels measured by Western blot analysis. Liquid chromatography–mass spectrometry (LC–MS) analysis of cerebellar tissue suggested that the behavioral and biochemical variability was due to the ad libitum administration route, as the mice ingested different amounts of the drinking water. The concentration of MB found in the brain correlated positively with MWM performance and inversely with soluble tau levels. A high brain concentration ($>$ 470 μM) was needed for effective therapeutic effect, which is worrisome because this dose is higher than the US Food and Drug Administration (FDA)-recommended dose for human use.[60]

Another study used a triple-transgenic (3×Tg) mouse model of AD harboring APP, presenilin-1 (PS1), and tau mutations[61] to evaluate the effect of MB on both Aβ and tau pathology. Following 16 weeks of oral treatment, soluble Aβ levels were reduced with no changes in insoluble Aβ. In contrast to the Rember studies, tau levels in treated and nontreated 3×Tg mice did not differ in this study.[62]

The different experimental parameters, animal models, and treatment course among the MB studies make comparison of the results and evaluation of the effect of MB on Aβ and tau aggregation in vivo difficult. Future experiments, for example, using Western blots and sandwich enzyme-linked immunosorbent assay (ELISA) to specifically measure levels of soluble and insoluble tau and Aβ[63] will be required for correlating the effect of this agent on assembly and toxicity.

O4

A recent study by Bieschke et al. discovered O4 (Figure 3), a small molecule related to the orcein dye that seems to share many of the in vitro characteristics of MB.[64] Membrane filter retardation assays (FRAs) and circular dichroism (CD) spectroscopy showed that O4 reduces the lag phase of Aβ42 polymerization, corresponding to increased formation of β-sheet-rich aggregates. Addition of Aβ42 monomers to amyloid fibrils increased ThT fluorescence, demonstrating that O4-treated fibrils were seeding competent. O4 promoted formation of long amyloid fibrils as opposed to protofibrils observed by TEM following incubation of Aβ42 under the same conditions in the absence of O4. In SDS-PAGE analysis, O4-treated Aβ42 displayed increased SDS-resistant large aggregates and decreased monomers and small oligomers relative to Aβ42 alone. O4-treated Aβ42 oligomers were unreactive with the A11 antibody. NMR and docking simulations suggested that O4 binds to the hydrophobic regions of Aβ42, particularly to hydrophobic binding pockets in Aβ42 fibrils.

O4 was found to inhibit Aβ42-induced toxicity in a dose-dependent manner using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Electrophysiological experiments using wild-type (WT) rat hippocampal slices were performed to measure long-term potentiation (LTP), an in vitro correlate of learning and memory. Inhibition of LTP by Aβ42 oligomers (50 μM) was abolished in the presence of O4 (10 mM). The study suggests that O4 accelerates the assembly pathway to prevent oligomer-induced toxicity. Future experiments are needed to test whether O4 yield the expected beneficial effects in vivo.

Ellagic acid

Another compound explored for its ability to promote fibrillization is the polyphenol, ellagic acid, which is found in many nuts and fruits, and similar to many polyphenols, possesses anti-inflammatory and antioxidant properties. An in vivo study was conducted first using pomegranate juice, which contains a high concentration of ellagic acid. The juice was diluted in the drinking water of transgenic mice expressing the Swedish mutant of human APP (Tg2576).[65] Following six months of treatment, the mice had reduced cognitive deficits, brain levels of soluble Aβ, and amyloid deposition as compared with a control group that received sugar water.[66] In vitro studies using TEM and CD spectroscopy showed that ellagic acid promotes Aβ fibrillization.[67] Using the single-chain variable fragment (scFv) oligomer-specific antibody W8,[68] it was found that oligomer levels decrease upon incubation with ellagic acid, and these observations correlated with decreased cytotoxicity measured by the MTT assay.[67] However, ellagic acid has been shown to possess low bioavailability and is rapidly eliminated from the body.[69] Such issues would need to be addressed before this compound can be considered a viable drug candidate.

Additional compounds

Additional compounds that might promote fibrillization include the peptide $KLVFFK_6$, which has been shown to increase the rate of Aβ aggregation and decrease its toxicity.[70] It was suggested that the peptide accelerates the rate of lateral association of protofilaments into fibrils that are more branched than firbils formed in the absence of this peptide.[71] In a different study examining multiple compounds for their effect on Aβ assembly, Necula et al. found that azure C, basic blue 41, and daunomycin promote in vitro fibrillization. The effect of these compounds on toxicity induced by Aβ or other amyloidogenic proteins is currently unknown.[72]

As mentioned earlier, aggregated α-syn and Htt could have a protective role against the toxicity inflicted by oligomers of these proteins.[40,41] To our knowledge, to date, only one compound, 5-[4-(4-chlorobenzoyl)-1-piperazinyl]-8-nitroquinoline (B2), has been found to accelerate the aggregation of a fragment of human Htt with 103 glutamines (Httex1-Q103) expressed in PC-12 cells and reduce proteasome dysfunction caused by transfecting CHO-K1 cells with Httex1-Q97.[73] Additionally, B2 reduced cytotoxicity of WT α-syn transfected in H4 neuroglioma cells, as measured by adenylate kinase release. The direct target of B2 is unknown, and the mechanism by which B2 exerts its effect on protein aggregation and toxicity is currently unclear. In another study, Kvam et al. utilized a human scFv antibody that specifically binds to Httex1 and enhances aggregation of the protein. However, when coexpressed with Httex1-Q72, the antibody increases cytotoxicity in immortalized rat striatal progenitor (ST14A) cells as measured by incubation with propidium iodide (PI).[74] Demonstration of direct binding of the antibody to Httex1-Q72 supports the authors' conclusion that molecules that accelerate aggregation might be detrimental to polyglutamine disorders. However, it is difficult to compare the studies using B2 and those using the scFV antibody directly as they utilized different cellular models and measurements of cytotoxicity.

Accelerating fibril formation is an intriguing therapeutic strategy based on the idea that reducing the steady-state concentration of toxic oligomers would decrease toxic insults. However, though many studies have disassociated fibril formation from toxicity, fewer studies have provided direct evidence linking drug-induced acceleration of fibril formation with decreased toxicity and neuroprotection.

Bodner et al. proposed that large aggregates could decrease the amount of toxic surface area or direct aberrantly folded proteins such as Htt and α-syn for destruction, thus leading to reduced neurotoxicity.[75] Although fibrils might be less toxic than oligomers, they still are unnatural, potentially harmful, species that the body tries to eliminate. Clearing mechanisms such as the ubiquitin–proteasome system (UPS) can be overstrained by accumulation of toxic species to be eliminated—a vicious cycle that leads to further toxicity.[76] In addition, a large body of research suggests that inflammation is closely associated with insoluble, fibrillar deposits of amyloidogenic proteins such as amyloid plaques, another hallmark of AD.[77] In this context, the fact that many of the compounds discussed above have pleiotropic effects is encouraging because their anti-inflammatory action might mitigate pro-inflammatory responses to increased fibril deposition. Nonetheless, whether or not acceleration of fibrillogenesis is a viable therapeutic strategy remains to be determined.

Strategy 3: Modulating the Pathway

If amyloidogenic proteins become toxic only when they undergo self-assembly, a reasonable strategy is to attempt to prevent the self-assembly altogether. In certain cases, this can be achieved by stabilizing the native structure, as discussed above. A more general approach is to disrupt the self-association process itself. Interestingly, an emerging observation is that compounds capable of disrupting this process do not completely prevent monomer to monomer association, but rather modulate the assembly process into formation of nontoxic structures. This appears to be the case both when the compounds affect the initial oligomerization by shifting the pathway towards nontoxic species, and when compounds disaggregate fibrils into nontoxic species. Ideally, a drug would

be capable of both preventing formation of toxic oligomers, and dissociating preformed aggregates into nontoxic, soluble structures that can be degraded by natural clearance mechanisms.

A large number of compounds have been studied as inhibitors/modulators of aberrant protein aggregation. We cannot cover all cases in this Minireview, and so the focus here is on promising compounds where the mechanism of action is believed to be targeting the aggregation pathway specifically. In addition, we attempt to cover mainly compounds for which multiple aspects of inhibitory activity have been assessed, including in vitro studies of: 1) inhibition of oligomerization and/or fibrillization, 2) formation of a nontoxic species, and 3) disaggregation of fibrils (Table 1). Studies in vitro for inhibition of cytotoxicity and/or synaptotoxicity, and/or in vivo for beneficial biochemical/histochemical effects and behavioral improvement are summarized in Table 2. Many of the compounds discussed below, especially the polyphenols, induce wide-ranging effects beyond their roles in the aggregation pathway. Although discussing these effects is beyond the scope of this Minireview, it is important to keep in mind that the possible multiple targets could enhance the therapeutic effect of the compound.

(−)-Epigallocatechine gallate

Several polyphenols originally explored for their antioxidant and anti-inflammatory activity also act as inhibitors/modulators of aberrant protein aggregation making them useful drug candidates as they could potentially act on multiple targets. A prominent example is the green-tea extract, (−)-epigallocatechine gallate (EGCG; for a review, see Ref. [78]). Ehrnhoefer et al. first began to elucidate the mechanistic details of the antiaggregation activity of EGCG with respect to mutant Htt. Atomic force microscopy (AFM) imaging with a glutathione S-transferase (GST)-tagged Httex1 fusion protein (GST-HDQ53) revealed that EGCG decreased the number of small HDQ53 oligomers and increased the number of larger oligomers.[79] A similar effect was observed with Aβ and α-syn.[80] Thioflavin T (ThT) fluorescence coupled with TEM analysis suggested that EGCG suppresses fibril formation and forms compact, spherical oligomers instead. SDS-PAGE and immunoblotting demonstrated that the oligomers are stable, HMW aggregates that do not disassemble under the denaturing effects of SDS. The oligomers were also found to be nontoxic in an MTT assay, they did not react with the A11 antibody in a dot blot assay, and they were seeding incompetent in a ThT assay. These results suggest that EGCG modulates the aggregation pathway of Aβ and α-syn to form nontoxic oligomers that do not convert into amyloid fibrils.[80]

Morphological analysis using AFM and TEM showed that EGCG remodeled α-syn and Aβ fibrils into unordered, amorphous aggregates.[81] CD spectroscopy and ThT fluorescence suggested that the compound remodeled the β-sheet conformation of the amyloid structures so that they no longer were seeding competent.[23] The disaggregated fibrils were SDS-resistant and nontoxic. Together, the data suggest that the disaggregated fibrils do not disassemble easily to release monomers or small oligomers that might reaggregate into toxic species.[81] This is further supported by NMR studies suggesting that EGCG directly interacts with the polypeptide main chain that is common to all proteins and thus is not sequence-specific, rendering EGCG an effective inhibitor for Htt, α-syn, and Aβ.[80] In accordance with this hypothesis, a recent study showed that EGCG also inhibits IAPP aggregation, prevents IAPP amyloid formation, disaggregates IAPP fibrils, and protects rat insulinoma (INS-1) β-cells from IAPP-induced toxicity.[82]

In vivo studies with EGCG have largely correlated with in vitro data.[79] To test the toxicity of the EGCG-stabilized Htt oligomers discussed above, Ehrnhoefer et al. performed studies in yeast overexpressing Httex1-Q72. Following treatment with EGCG (500 μM), the mutant yeast cells demonstrated significant growth and harbored less protein aggregates as observed by fluorescence microscopy and membrane FRAs. Studies were also conducted using a fly model of HD overexpressing Httex1-Q93 protein. Transgenic flies fed with EGCG from embryogenesis onward exhibited a dose-dependent decrease in photoreceptor neurodegeneration and motor dysfunction. Taken together, the in vitro and in vivo experiments suggest that EGCG treatment led to formation of large, spherical Htt oligomers and decreased the toxicity induced by the Httex1-Q72-transgene product in yeast and Httex1-Q93 in flies.[79]

EGCG has also demonstrated beneficial effects in Tg2576 mice.[65] In a first set of experiments by Rezai-Zadeh et al., 12-month-old mice injected intraperitoneally (i.p.) with EGCG (20 mg kg^{-1}) for 60 days showed significant reduction in Aβ deposits and in soluble and insoluble Aβ40 and Aβ42 as measured by IHC and ELISA, respectively.[83] In a follow-up study, EGCG (50 mg kg^{-1}) was administered ad libitum in the drinking water of Tg2576 mice for six months.[84] As a result, the mice exhibited reduced levels of Aβ and soluble hyperphosphorylated tau, although total phosphorylated tau levels did not change. Both i.p. and oral administration of EGCG induced significant improvement in working memory, measured by the radial arm water maze, although improvement was more pronounced in mice treated by i.p. administration than in those treated orally.[84]

The potential of EGCG as a drug candidate has been discussed in a comprehensive review,[85] which considered the safety and dose/administration of this agent in reports of clinical trials for non-neurodegenerative diseases. The review suggests that although the bioavailability of EGCG is low and erratic, recently developed delivery methods could improve this issue. In addition, at 100 mg kg^{-1}, EGCG was found to be lethal in mice, and the doses needed for efficacy could be dangerously close to this lethal dose.[86] Clinical trials to evaluate EGCG as a potential therapeutic for neurodegenerative diseases are still in progress (see http://clinicaltrials.gov). The data accumulated thus far suggest that EGCG is a promising compound for the treatment of amyloid-related diseases. A potential caveat in developing EGCG further is that the molecular basis for the high affinity of EGCG for amyloidogenic proteins is unknown. However, it is possible that this will not be a problem and development will be successful despite the lack of mechanistic understanding.

Table 1. Protein assembly effects of compounds whose proposed mechanism of action is to modulate the assembly pathway toward nontoxic oligomers/aggregates.[a]

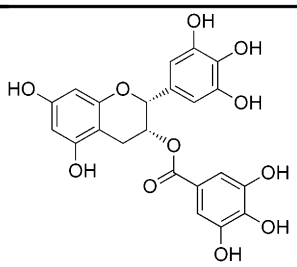
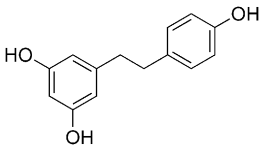
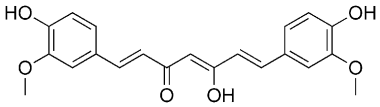
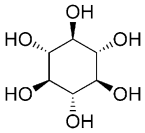
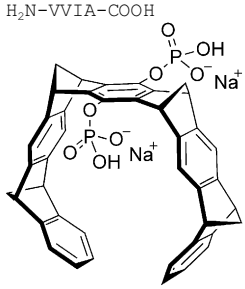
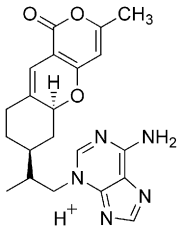
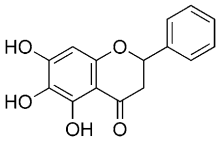
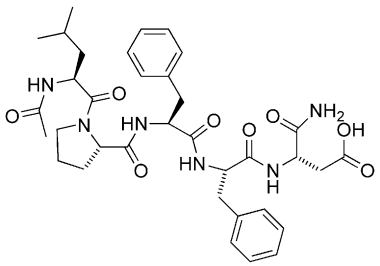
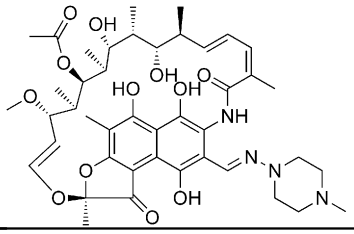
Compound	Structure	Inhibition of fibril formation	Stabilization of nontoxic oligomers	Fibril dissocation
EGCG		mutant Htt[79] Aβ[80] α-syn[80] IAPP[82]	mutant Htt[79] Aβ[80] α-syn[80] IAPP[82]	Aβ[80] α-syn[80] IAPP[82]
Resveratrol		Aβ[87–88]	Aβ[87,88]	Aβ[87,88]
Curcumin		Aβ[96] α-syn[97,99] mutant Htt[102]	Aβ[96]	Aβ[96] α-syn[97,101]
scyllo-inositol		Aβ[112]	Aβ[112]	unknown
D3	H_2N-rprtrlhthrnr-COOH[b]	Aβ[127,128]	Aβ[128]	unknown
Aβ(39–42)	H_2N-VVIA-COOH	Aβ[126]	Aβ[122–124]	No
CLR01		Aβ[130] tau[130] α-syn[132] IAPP[130] calcitonin[130] TTR[130] β_2-microglobulin[130] insulin[130]	Aβ[130] α-syn[132]	Aβ[130] α-syn[132] IAPP
C2		Aβ[135] mutant Htt[136]	unknown	Aβ[137]
Baicalein		Aβ[138] α-syn[138,139]	unknown	Aβ[138] α-syn[139a]
iAβ5p		Aβ[140]	unknown	Aβ[140]

Table 1. (Continued)

Compound	Structure	Inhibition of fibril formation	Stabilization of nontoxic oligomers	Fibril dissocation
Rifampicin		Aβ[141] α-syn[142] **IAPP**[143] **mutant Htt**[144]	unknown	α-syn[142] **IAPP**[143]

[a] Gray boldface text indicates an insignificant or negative effect of a compound. [b] Lower-case letters indicate D-amino acids.

Resveratrol

Table 2. Biological effects of compounds whose proposed mechanism of action is to modulate the assembly pathway toward nontoxic oligomers/aggregates.[a]

	Cell culture		Animal models		Human studies
Compound	Inhibition of cytotoxicity	Inhibition of synaptotoxicity	Decrease of pathological deficits	Decrease of behavioral deficits	Clinical trials
EGCG	Aβ[80] α-syn[80] IAPP[82]	unknown	Htt aggregates[79] Aβ plaques[83] soluble Aβ[83] insoluble Aβ[83] p-tau[84]	Motor function[79] Cognitive function[83,84]	in progress
Resveratrol	Aβ[87–88]	unknown	Aβ plaques[90]	unknown	in progress
Curcumin	~αsyn[101] **mutant Htt**[102]	Aβ[103,107]	Aβ plaques[96,107] insoluble Aβ[96,104] soluble Aβ[104]	cognitive function[103]	**cognitive deficits**[145]
scyllo-inositol	Aβ[112]	Aβ[117]	Aβ plaques[115–116] insoluble Aβ[115] soluble Aβ[115]	cognitive function[115,117,118]	**cognitive deficits**[120]
D3	Aβ[127]	unknown	Aβ plaques[128]	cognitive[128]	none conducted
Aβ(39–42)	Aβ[122,123]	Aβ[122]	unknown	unknown	none conducted
CLR01	Aβ[130] α-syn[132] IAPP[130] calcitonin[130] TTR[130] β_2-microglubin[130] insulin[130]	Aβ[133]	Aβ plaques[133] p-tau[133] α-syn[132]	unknown	none conducted
C2	Aβ[135,146]	unknown	insoluble Aβ[137] soluble Aβ[137]	unknown	none conducted
Baicalein	Aβ[138] α-syn[138,147]	unknown	unknown	unknown	none conducted
iAβ5p	Aβ[140]	Aβ[148]	Aβ plaques[148] soluble Aβ[148]	cognitive function[149]	none conducted
Rifampicin	Aβ[141,150]	unknown	soluble α-syn[151] insoluble α-syn[151]	unknown	none conducted

[a] A tilde (~) indicates conflicting effects. Gray boldface text indicates an insignificant or negative effect of a compound.

Another polyphenol with a broad range of neuroprotective and antiamyloidogenic activities, similar to EGCG, is resveratrol, a main constituent of grape seeds. Many studies have demonstrated a beneficial neurological effect of grape seed extract or modest red wine intake. However, to specifically elucidate the mechanism of action of resveratrol on protein aggregation, it has been essential to study purified resveratrol.

Resveratrol has been examined mainly for its effect on Aβ. Concurrent ThT fluorescence, CD measurements, and TEM imaging by Feng et al. suggested that resveratrol prevents Aβ42 fibrillization and β-sheet formation.[87] Instead of fibrils, resveratrol-treated Aβ forms a large amount of oligomers. Resveratrol was also shown to disaggregate preformed Aβ fibrils into oligomers. These oligomers react with antibodies A11 and W8, which have been shown to recognize toxic Aβ oligomers, but resveratrol increased the cell viability in a dose-dependent manner as measured by the MTT assay,[87] suggesting that A11 and W8 might not be able to distinguish between toxic and nontoxic oligomeric structures. Ladiwala et al. further investigated the ability of resveratrol to modify both prefibrillar oligo-

mers and fibrils. Their results suggest that following treatment with resveratrol, both prefibrillar oligomers and fibrils form large, insoluble HMW aggregates that, consistent with the previous study, are nontoxic.[88] Both studies speculated that resveratrol could bind to Aβ42 by aromatic packing and hydrophobic forces, and thus modify the protein conformation subtly in a manner that produces nontoxic structures, which are still detectable by oligomer-specific antibodies.

Extensive in vivo studies have examined the neuroprotective effects of substances that contain resveratrol. For example, in a study evaluating the effect of the compound on brain protein aggregation, six- and ten-month-old Tg2576 mice were administered grape seed extract in the drinking water for five months.[89] Biochemical and IHC analysis in the ten-month-old mice showed that HMW Aβ oligomers, Aβ40, Aβ42, and plaque load were all reduced upon treatment. The decrease in HMW Aβ oligomers (detected by A11 antibody) corresponded with an increase in Aβ monomers (6E10 antibody), supporting the hypothesis that preventing Aβ oligomerization leads to an increase in monomers, which can be removed by clearing mechanisms. Following five months of treatment, behavioral tests on six-month-old mice showed cognitive improvement in MWM performance.[89] Nonetheless, the observation that the treatment led to a decrease in A11-reactive oligomers in the brains of the mice, when in vitro studies did not find an effect of resveratrol on such oligomers, suggests that the beneficial effect in mice might have been caused by mechanisms other than the effect of resveratrol on Aβ assembly. Because the study used grape seed extract, these effects could not be ascribed solely to resveratrol. To our knowledge, only one study has assessed the effect of pure resveratrol on Aβ aggregation in vivo.[90] In this study, 45-days-old Tg19959 mice expressing human APP695 with two familial AD-causing mutations (KM670/671NL and V717F)[91] were fed resveratrol-supplemented chow (300 mg kg^{-1}) for 45 days. IHC analysis demonstrated reduced plaque pathology in the medial cortex, striatum, and hypothalamus. However, plaque reduction was not statistically significant in the hippocampus. For a more complete picture, the effect of resveratrol on soluble and insoluble Aβ levels, plaque load, and learning and memory should be addressed in future studies.

Multiple studies have evaluated the role of resveratrol in neuroprotection by focusing on aspects other than protein aggregation. Granzotto et al. suggested that based on the available studies, the neuroprotective effect of resveratrol was mainly due to its antioxidant and anti-inflammatory activities rather than its aggregation-modulating properties.[92] Regardless of the mechanism of action, several clinical trials of resveratrol for neurodegenerative disorders are currently in progress (http://clinicaltrials.gov). Due to its pleiotropic qualities, the safety and bioavailability of resveratrol have also been assessed in several clinical trials for amyloid-unrelated diseases.[93] The limited bioavailability of resveratrol and its rapid clearance from the body heighten the need to stabilize it by better formulation and/or improved analogues.[94] A clinical trial using a capsule form of resveratrol in myeloma patients has recently been discontinued due to a high occurrence of kidney failure. Several experts proposed that the kidney failure might have occurred due to dehydration, as a high dose of resveratrol caused side effects such as nausea and vomiting.[95] These results raise concerns about the ability to use resveratrol efficaciously for amyloid-related disease in doses that do not cause serious side effects.

Curcumin

A polyphenol with particularly interesting effects on protein aggregation is curcumin, a compound isolated from tumeric. Yang et al. performed in vitro experiments utilizing TEM imaging and ELISA revealing that curcumin inhibits Aβ fibril formation and disaggregates preformed fibrils.[96] Importantly, when incubated with Aβ monomers (0, 4, or 16 μM of curcumin with 5 μM of Aβ42), dot blot and Western blot analyses with the A11 antibody suggested that curcumin blocks oligomer formation in a dose-dependent manner and increases the amount of monomers. Unlike with EGCG and resveratrol, detailed structural analysis of the species formed upon interaction of curcumin with Aβ has not been reported to our knowledge. However, curcumin was shown to block Aβ42-induced toxicity when applied to SH-SY5Y neuroblastoma cells.[96]

Experiments examining the effect of curcumin on α-syn aggregation have yielded complex results. Using ThT fluorescence and TEM, Ono et al. demonstrated that the compound inhibits fibrillization and destabilizes fibrils of α-syn.[97] Pandey et al. performed Western blots with antibody Syn-202, which binds aggregated α-syn,[98] and showed that curcumin incubation increases soluble α-syn levels and retards the formation of HMW aggregates in a dose-dependent manner.[99] A recent study using fluorescence quenching of α-syn, which was modified to contain single tryptophan and cysteine residues, has suggested that curcumin binding increases the rate of intramolecular reconfiguration, concomitantly decreasing intermolecular iteractions and aggregation, yet this mechanism could be unique to the interaction of curcumin with α-syn.[100] Unfortunately, these studies did not assess the toxicity of the species formed upon interaction of α-syn with curcumin.[101]

Wang et al. found that curcumin incubation with oligomeric α-syn decreased toxicity as assessed by the lactate dehydrogenase (LDH) assay in SH-SY5Y cells.[101] Interestingly, curcumin-induced destabilization of preformed α-syn fibrils, monitored by AFM imaging, resulted in a significant increase in toxicity, suggesting that the incubation of curcumin with α-syn fibrils might lead to accumulation of a toxic species.

Concerning results have also been observed in a study assessing the effect of curcumin on N2a cells expressing a polyQ-expanded truncated N-terminal fragment of Htt (NT-Htt-Q150). Following exposure of the cells to different doses of curcumin for up to 10 h, fluorescence microscopy revealed a dose- and time-dependent increase in NT-Htt-Q150 aggregation and cell death relative to cells not treated with curcumin. It was proposed that the compound might induce proteasomal dysfunction as proteosome activity decreased upon increasing doses of curcumin.[102] This result supports the study described in the Strategy 2 section in which enhancing Htt aggregation

by an scFV antibody led to increased cell death, and contradicts a study using compound B2 by Bodner et al., which suggested that Htt aggregation is neuroprotective.[73,74] Thus, it is still largely unclear how aggregation of Htt is related to neurotoxicity.

Thus far, in vivo studies with curcumin have focused mainly on AD. After five months of ingesting curcumin-supplemented chow ($\delta = 500$ ppm), 17-month-old Tg2576 mice showed significant reduction of plaque burden and insoluble Aβ levels—a promising result suggesting that curcumin has beneficial effects on brain pathology, though behavioral experiments are needed to assess whether these biochemical data correlate with cognitive improvements.[96] In another study, two doses of curcumin were fed to WT Sprague–Dawley (SD) rats intracerebroventricularly (i.c.v.) infused with lipoprotein carrier-associated Aβ40 and Aβ42 to induce neurodegeneration. A curcumin dose (δ) of 2000 ppm fed to 22-month-old rats for one month suppressed synaptophysin loss as measured by ELISA. A lower dose ($\delta = 500$ ppm) prevented spatial memory deficits in the MWM and reduced both Aβ deposits measured by IHC and postsynaptic density (PSD)-95 loss assessed by Western blot.[103] Intriguingly, a study examining the effect of curcumin administered orally for six months in ten-month-old Tg2576 mice found that curcumin decreased soluble and insoluble Aβ levels only at a low dose ($\delta = 160$ ppm), whereas a high dose ($\delta = 5000$ ppm) did not lead to significant differences.[104]

Garcia-Alloza et al. used multiphoton microscopy (MPM), which allows in vivo examination of the effect of curcumin on existing amyloid plaques by tagging and tracking the plaques for seven days.[105] This technique was applied to eight-month-old APPswe/PS1ΔE9 mice, which harbor familial AD mutations in both APP and PS1.[106] Treatment with curcumin (7.5 mg kg^{-1} day^{-1}) administered intravenously (i.v.) resulted in fewer and smaller plaques and significant recovery of morphological abnormalities and dystrophic swelling in neurites. Surprisingly, there was also a significant increase in the ratio of soluble Aβ42/40, which is a marker for AD. The authors suggested that this could be a beneficial result because clearing mechanisms could be more effective on soluble proteins than on insoluble aggregates.[107] It would be interesting to correlate such a study with behavioral analysis to explore the relationship between the increase in the ratio of soluble Aβ42/40 and changes in learning and memory.[105]

The overall effect of curcumin on the amyloidogenic pathway proves to be complex. In particular, in vitro studies suggest that unlike EGCG, curcumin might be sequence-specific rather than structure-specific, as its actions differ depending on the amyloidgenic protein in question. Because studies in animal models of AD have shown beneficial effects, curcumin has been assessed for its effectiveness in clinical trials for AD. Toxicity studies indicated that curcumin is safe at relatively high doses, yet water solubility and bioavailability issues need to be resolved (for a review, see Ref. [108]). A pilot study with AD patients found no significant improvement in the mini-mental state examination (MMSE).[109] A phase II trial showed no significant improvement in the MMSE, ADAS-Cog, neuropsychiatric inventory (NPI), and Alzheimer's disease cooperative study–activities of daily living (ADCS-ADL) scores. There was also no significant differences in plasma or CSF biomarkers of Aβ40, Aβ42, total tau, and p-tau.[110] Both of these studies had relatively short duration periods (5.5–6 months). A longer (24-months), ongoing, early intervention study is expected to assess the effects of the compound over an extended period of time (see http://clinicaltrials.gov for more details of this trial; Trial number NCT00595582).

Scyllo-inositol

The strategy 3 compounds discussed above are polyphenols from natural sources, which possess wide-ranging antioxidant and anti-inflammatory effects.[111] Hereafter, we discuss compounds distinct from this group, which act via a similar modulation strategy. One such compound is *scyllo*-inositol, one of nine stereoisomers of inositol, a sugar commonly found at the headgroup of endogenous phosphatidylinositol lipids. An early ThT fluorescence and CD spectroscopy study by McLaurin et al. demonstrated that whereas phosphatidylinositol induces Aβ fibrillization, *myo*-inositol inhibits Aβ fibrillization.[112] A follow-up study assessed the effect of other inositol stereoisomers on Aβ fibril formation using ThT fluorescence, TEM, and Aβ-induced cytotoxicity measured by an LDH release assay.[113] In this study, *epi*- and *scyllo*-inositol were found to inhibit Aβ42 fibril formation and induce formation of β-structured, nonfibrillar complexes. These two inositol isomers were also found to inhibit Aβ42-induced cytotoxicity. Further studies showed that binding to Aβ is highly sensitive, not only to the stereochemistry of *scyllo*-inositol[113] but also to small chemical modifications.[114]

McLaurin et al. went on to study the effect of *scyllo*- and *epi*-inositol on TgCRND8 mice, which, similar to Tg19959 mice, harbor a double-mutant form of APP695 (KM670/671NL and V717F).[91] A four-month prophylactic administration of either inositol isomer to six-week old mice resulted in significant cognitive improvement in the MWM and decreased Aβ plaques and both soluble and insoluble Aβ40 and Aβ42.[115] After six months of treatment, *scyllo*-inositol had a stronger effect in reducing Aβ brain levels than *epi*-inositol. Both isomers ameliorated astrogliosis, synaptophysin levels, and survival rates, and again *scyllo*-inositol had a stronger effect. Notably, *scyllo*-inositol induced these effects not only following six months of treatment of six-week-old mice, but also when administered for 28 days to five-month-old mice that already exhibited substantial AD-like pathology, including increased Aβ levels and plaque distribution.[115] This suggests that the compound might be useful both for prevention and treatment of AD, because it putatively acts on both ends of the aggregation pathway, though no in vitro study has specifically assessed the ability of *scyllo*-inositol to disaggregate fibrils. The hypothesis that the mechanism of action of *scyllo*-inositol involves clearance of Aβ oligomers was substantiated in this study as Aβ oligomers levels (probed with unidentified oligomer-specific antibodies) were reduced upon *scyllo*-inositol treatment.[115] In a follow-up study, *scyllo*-inositol was administered in the drinking water of five-month-old TgCRND8 mice for two months. Plaques of all sizes were reduced following the treatment, leading Fenili

et al. to suggest that *scyllo*-inositol inhibits plaque growth by intercalating into the β-structure of aggregates as well as "capping-off" the growing ends of the aggregates. An observed decrease in both soluble and insoluble Aβ levels suggests that clearance of Aβ was facilitated by the treatment.[116]

Scyllo-inositol was also found to be a potent inhibitor of synaptotoxicity as assessed by LTP measurements in WT hippocampal neurons. *Scyllo*-inositol (1.23 μM) pretreatment of Aβ-rich conditional media (CM) from CHO cells stably expressing human APP V717F (7PA2) demonstrated rescue of LTP inhibition by the 7PA2 CM, which had been shown previously to contain toxic Aβ oligomers.[117] 7PA2 CM was further fractionated using SEC to separate soluble Aβ species by molecular weight. Affinity-based separation using *scyllo*-inositol attached to epoxy resin resulted in preferential pull-down of Aβ trimers, suggesting that *scyllo*-inositol exerts its inhibitory activity through direct binding to Aβ trimers.[118] Additional behavioral studies were conducted on normal adult rats two hours after a five-minute i.c.v. injection with 7PA2 CM. Incubation of 7PA2 CM with 5 μM *scyllo*-inositol prior to injection completely abolished behavioral deficits measured in an alternating lever cyclic ratio (ALCR) assay. Similar results were obtained with rats receiving i.c.v. injection of 7PA2 CM that had been administered *scyllo*-inositol (30 mg kg^{-1} day^{-1}) in their drinking water for five days, further confirming the efficacy of the compound in vivo.[118]

Inositols have been used to treat various psychiatric disorders. Thus, safety issues and BBB penetration have already been established, although these studies did not assess specifically the *scyllo* stereoisomer.[119] An ongoing clinical trial exploring the effect of *scyllo*-inositol in patients with mild-to-moderate AD revealed no significant effects, and nine deaths were reported in the groups receiving the two highest doses (1000 and 2000 mg twice daily), following which these doses were discontinued. The investigators suggested that the sample size was too small for establishing cognitive benefits measured by the neuropsyhological test battery (NTB) and ADCS-ADL scale.[120]

C-terminal fragments of Aβ42

Polyphenols and inositols are naturally produced compounds that have been shown, primarily through empirical findings, to modulate protein assembly and are appealing because they are known to be safe. However, we largely do not understand why or how these compounds modulate the assembly, making their further development into efficacious drugs difficult. A different class of compounds comes from synthetic laboratories and is based on rational considerations of the biophysical and biochemical properties inherent in protein aggregation and the molecular interaction controlling the process.

Our laboratory has postulated that C-terminal fragments (CTFs) of Aβ42 might compete with the self-assembly of full-length Aβ42, and thereby disrupt its oligomerzation and inhibit its toxicity. The rationale for this approach was based on evidence demonstrating that the C terminus distinguishes between the assembly processes, and by inference the toxicity, of Aβ40 and Aβ42.[25b,121] Studies testing the optimal length of Aβ42 CTFs demonstrated that nearly all the CTFs enhanced cell viability in MTT and LDH assays.[122] Aβ(31–42), Aβ(39–42), and unexpectedly Aβ(30–40), which was used as a control, exhibited the most robust protection from Aβ42-induced neurotoxicity and were evaluated further for their ability to protect hippocampal neurons from Aβ-induced reduction of spontaneous miniature excitatory postsynaptic currents (mEPSCs). The mEPSC frequency was rescued significantly when neurons were perfused with the Aβ42–CTFs mixture relative to Aβ42 alone.[122–123] Mechanistic studies using dynamic light scattering (DLS), photoinduced cross-linking of unmodified proteins (PICUP), and discrete molecular dynamics (DMD)[122–124] suggested that CTFs coassemble with Aβ42 into heterooligomers and cause an increase in abundance of two distinct oligomer assemblies that are populated to a substantially lower extent than by Aβ42 alone. The increase in abundance of principally the smaller of the two oligomer populations correlated with retardation of fibril growth, particularly for the longer CTFs.[122,123] DMD simulations suggested that these CTFs, and particularly Aβ(39–42), bound to the N terminus and reduced the solvent accessibility in this region of Aβ42.[124] These predictions were supported independently in a study using intrinsic fluorescence and solution-state NMR,[125] and by ion mobility-mass spectrometry (IM-MS) experiments combined with replica-exchange molecular dynamics.[126] Intriguingly, the latter study revealed that Aβ(39–42) binding shifts the oligomer size frequency distribution of Aβ42 from dodecamers to smaller oligomers, yet does not prevent Aβ42 fibril formation. This result provided strong support to the notion that oligomers are more toxic than fibrils.[126] These recent studies present novel peptides that hold promise as therapeutic agents, though structural modification rendering them metabolically stable will likely be needed before in vivo studies can be conducted.

D3

Using mirror image phage display, Willbold and co-workers have identified a promising peptide inhibitor (D3), which comprises 12 D-amino acid residues.[127] ThT fluorescence and fluorescence correlation spectroscopy (FCS) assays demonstrated that D3 prevents Aβ aggregation.[127] D3 was also shown to disaggregate Aβ fibrils and was found to prevent Aβ-induced cytotoxicity, measured by an MTT assay. A battery of in vitro assays including DLS, SEC, turbidity, and TEM suggested that solutions containing Aβ mixtures with D3 form large particles with amorphous morphology. HMW fractions were seen when the Aβ–D3 mixtures were fractionated by density gradient centrifugation, and SDS-PAGE followed by silver staining.[128] Computational studies led the group to propose that D3 forms strong interactions with negatively charged groups in Aβ to reduce solubility and promote aggregation.[128] APPswe/PS1ΔE9 mice unilaterally infused directly into the hippocampus using pumps containing D3 (0.5 mg at 6 μL day^{-1}) or fed D3 in drinking water (0.5–1 mg day^{-1} depending on water consumption) for eight weeks both showed significant reduction

of Aβ plaque load and cognitive deficits as measured by the MWM test.[128] Future studies addressing D3's effect on soluble and insoluble Aβ levels would provide a clearer correlation between in vivo and in vitro studies concerning the peptide's mechanism of forming large HMW aggregates to ameliorate toxicity.

Molecular tweezers

Recently, our group discovered that water-soluble "molecular tweezers" (MTs) specific for lysine[129] were general inhibitors of aggregation and toxicity of amyloid proteins, including Aβ, tau, α-syn, IAPP, and others (Table 1).[130] MTs modulate the amyloidogenic pathway in a novel process-specific manner targeting both hydrophobic and Coulombic interactions involving lysine, which are important in the initial assembly process of amyloid proteins.[121c,131] MTs bind to lysine with a moderate affinity, presumably allowing them to disrupt the relatively weak interactions that mediate the initial nucleation/oligomerization steps in amyloid assembly without affecting normal protein function.

Sinha et al. demonstrated using ThT fluorescence, turbidity, and TEM assays that an MT derivative termed CLR01 inhibits fibril formation by multiple different amyloidogenic proteins. The toxicity inflicted by these proteins to neuronal or pancreatic cells was inhibited as shown by an MTT assay.[130] In most cases, complete inhibition of aggregation was achieved at a 1:1 ratio of protein to CLR01. Supporting the proposed mechanism of action, CLR01 induced mild toxicity at concentrations two- to three-orders of magnitude above those required for inhibition of aggregation or toxicity in vitro. Binding studies using mass spectrometry coupled with electron capture dissociation and solution-state NMR indicated that CLR01 bound to Aβ monomers already at a 10:1 ratio of Aβ to CLR01, with the main binding sites at the two lysine residues on Aβ. Dot blot experiments with antibody A11 revealed that CLR01 modulates Aβ42 oligomerization such that A11 immunoreactivity was not observed even at the onset ($t=0$). DLS experiments demonstrated that CLR01 stabilizes oligomer populations similar to those observed in the presence of Aβ42 CTFs[123] and prevents Aβ fibril formation. The dot blot, DLS, TEM, and NMR experiments, in conjunction with MTT assays, demonstrated that CLR01 bind to Aβ rapidly, already at the monomer stage, and stabilizes nontoxic oligomers. Moreover, using ThT fluorescence and TEM, CLR01 was also found to disaggregate Aβ40, Aβ42, α-syn, and IAPP fibrils.[130,132]

In primary neuronal cultures, CLR01 was protective against the toxic effect of Aβ42 on dendritic spine density and morphology.[133] In addition, disruption of basal synaptic activity and LTP by Aβ42 was rescued significantly by CLR01. In initial mouse studies, CLR01 was administered to 15-month-old 3× Tg mice at 40 $\mu g\,kg^{-1}\,day^{-1}$ via subcutaneous miniosmotic pumps for 28 days. The treatment resulted in a significant decrease in Aβ plaque load and concomitant reduction of p-tau and microgliosis levels. Importantly, the mice treated with CLR01 did not show adverse effects.[133]

CLR01 has also been assessed in a novel zebrafish model expressing human WT α-syn in neurons. Expression of α-syn caused severe deformation and early mortality of zebrafish embryos.[132] Addition of CLR01 to the water in which the embryos developed led to a dramatic improvement in phenotype and viability. The study also found that CLR01 treatment maintained α-syn in a soluble form allowing its degradation by the UPS, which is known to be impaired by α-syn oligomers and aggregates,[134] suggesting that the labile binding of CLR01 to lysine residues inhibits α-syn aggregation but not ubiquitination. Future animal studies in models of AD and PD need to assess levels of insoluble and soluble Aβ, tau, and α-syn, respectively, upon treatment, as well as behavioral measures of each disease.

Conclusions

Many additional compounds have been reported to modulate the aggregation pathway of amyloidogenic and are not discussed in more detail here due to space limitations. Some of these compounds are listed in Tables 1 and 2. The discovery of multiple compounds and the detailed studies reviewed above represent significant progress towards disease-modifying therapeutic tools for prevention and treatment of amyloid-related disorders. Nonetheless, in most cases, understanding of how these compounds inhibit the toxicity of amyloidogenic proteins will require both understanding of the mechanisms of toxicity themselves, and further characterization of the binding between the inhibitory/modulatory compounds and the offending proteins. For example, it is important to characterize simultaneously disaggregated and modulated protein assemblies using spectroscopic and morphologic techniques, toxicity assays, and assembly-specific antibodies to decipher the relationships among conformation, aggregation/oligomerization state, and toxic activity. In addition, to establish efficacy, animal experiments should consistently examine both pathologic and cognitive effects.

Outlook

A confounding issue with many drug candidates is that their mechanisms of action are poorly understood even at the level of in vitro experiments. As a result, studies involving more complex cellular and whole-animal systems often lead to further complication and discrepancy, and correlating studies that use different systems or are performed by different research groups is difficult. Many compounds influence the aggregation pathway of amyloidogenic proteins in distinct ways. Interestingly, compounds as diverse as polyphenols, sugar derivatives, peptides, and artificial receptors, such as molecular tweezers, bind directly to different amyloidogenic proteins and often yield similar effects on the aggregation pathways, whereas in other cases, compounds with similar structures exert opposite effects, such as inhibition or acceleration of fibril formation. Developing new methods for probing molecular interactions and deciphering the mechanisms by which inhibitors and modulators exert their effects will be crucial for generation of

new avenues towards therapy for diseases caused by aberrant protein folding and aggregation.

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