Small molecule inhibitors of A-aggregation and neurotoxicity

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Research Article

Small Molecule Inhibitors of Aβ-Aggregation and Neurotoxicity

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ABSTRACT Alzheimer disease (AD) is characterized pathologically by extracellular amyloid deposits composed of Aβ peptide, neurofibrillary tangles (NFTs) made up of hyperphosphorylated tau, and a deficit of cholinergic neurons in the basal forebrain. Presently, only symptomatic therapies are available for the treatment of AD and these therapies have a limited time frame of utility. Amyloid disorders represent the effects of chronic Aβ production and are not a secondary pathological effect caused by a distant trigger; therefore targeting Aβ is a viable pursuit. In this review, we will discuss the various small molecule anti-aggregation inhibitors that have been reported in the literature, with emphasis on compounds that are presently being investigated in clinical trials. Drug Dev Res 70:111–124, 2009.

Key words: amyloid; aggregation; inhibitors; Alzheimer's disease

INTRODUCTION

An enormous amount of evidence, much of which materialized from analyzing hereditary forms of Alzheimer's disease (AD), share the consensus that Aβ aggregation is important in disease propagation [Hardy and Selkoe, 2002]. However, controversy still exists as to whether the fibrils are indeed a cause or a consequence of the disease. Aβ aggregation is an intricate process and appears to entail more than a simple conversion of soluble monomer to fiber. In addition, despite many mutations in the amyloid precursor protein (APP) gene associated with early onset of AD, the levels of amyloid deposited in the brain do not equate with disease severity. A likely explanation for the occurrence is that the β-structured prefibrillar species, soluble amyloid oligomers or prefibrillar aggregation intermediates, are the primary toxic species in degenerative amyloid diseases (Fig. 1) [Glabe, 2005; Hardy and Selkoe, 2002]. Most importantly, it is clear that the most crucial factor determining Aβ toxicity is the aggregation state. Further, it remains to be determined whether the oligomers that appear transiently at different stages during fibrillization are simply intermediates on the pathway leading to fibril formation, or whether they represent "off pathway" aggregates that populate an

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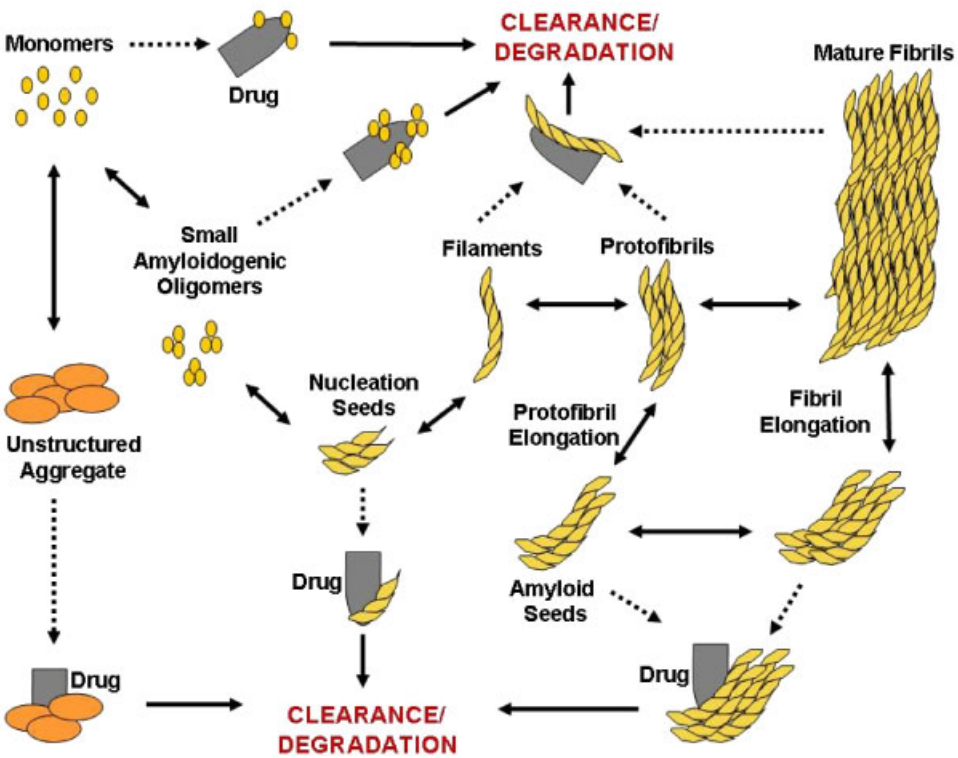

Fig. 1. Summary of amyloid assembly mechanism. In disease states, proteins that normally exist as soluble monomers undergo aggregation to form various intermediates consisting of unstructured aggregates or amyloidogenic species. Amyloidogenic oligomers subsequently assemble to generate insoluble fibrils that accumulate in the affected tissues or organs. Compounds that inhibit formation of these undesirable species may, therefore, be capable of protecting tissues or organs from their toxic effects. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com]

alternative aggregation pathway [Gorman et al., 2003]. If Aβ oligomers are simply intermediates on the pathway to fibril formation, then inhibitors that prevent oligomer formation would also be expected to prevent amyloid formation. However, if fibrils and oligomers represent distinct aggregation pathways, then some inhibitors would block Aβ oligomerization but not necessarily fiber formation or vice versa. Using an oligomer-specific antibody, A11, to detect oligomer formation, Necula and workers [Necula et al., 2007] investigated the mechanisms for action of a large panel of small molecules that had previously been reported to inhibit the aggregation and toxicity of different amyloidogenic proteins. The A11 antibody recognizes Aβ oligomers and protofibrils greater than 40 KDa in size but does not react with monomeric Aβ, fibrillar Aβ, or the amyloid precursor protein [Kayed et al., 2003]. Depending on the ability of the small molecules to modulate $A\beta_{42}$ aggregation into oligomers and/or fibers, the compounds were divided into three subsets. These data indicated that soluble oligomers are not obligate intermediates for fibril formation and that oligomers and fibrils belong to two distinct aggregation pathways. It is worthwhile to note that this does not necessarily suggest that oligomers do not ultimately form fibrils, as there is possibly more than one pathway leading to Aβ fibril formation. Oligomers may also constitute an "off pathway" assembly state whereby they themselves do not necessarily convert into fibrils but are present to maintain the concentration of monomers that in due course convert into fibrils.

Aggregation of Aβ initiates a series of events that ultimately results in neuronal death, as well as cognitive and behavioral decline that is characteristic of AD. Consequently, compounds that inhibit Aβ aggregation, fibrillization, and/or plaque formation may be capable of protecting neurons from Aβ toxicity and thus display therapeutic potential for the disease. For these reasons, a quest began to discover small molecules that may intercede with the in vitro or in vivo aggregation and/or neurotoxicity of Aβ peptides.

SYNTHETIC PEPTIDES, β-SHEET BREAKER PEPTIDES

D-Analogues and Disrupting/Recognition Elements

Because Aβ is self-assembling, the first strategy in developing Aβ aggregation inhibitors was to target the peptide sequence itself by using short peptide fragments homologous to the full-length wild-type protein.

In the hopes of developing a lead compound against amyloid formation, one of the first groups to make use of a homologous section of Aβ as a structural starting element was Tjernberg et al. who identified Aβ (16–20) (KLVFF), which bound to the full-length Aβ and prevented assembly into fibrils [Tjernberg et al., 1996]. Inhibition of aggregation by this small Aβ fragment was controlled through recognition of KLVFF to the identical sequence within full-length Aβ via hydrophobic and electrostatic interactions [Watanabe et al., 2001]. Slightly longer peptides containing this sequence or those with D-amino acid analogues, lflrr and yflrr, were subsequently synthesized and inhibited fibril formation [Tjernberg et al., 1997, 1998].

Simultaneously, Soto and co-workers also began work on peptide inhibitors aimed at the core region of Aβ, specifically residues 17–21 (LVFFA) [Soto et al., 1996, 1998]. Peptides with partial homology to the central 17–21 region of Aβ but with proline replacements at key positions were observed to convert Aβ fibrils to amorphous aggregates and inhibit Aβ toxicity in vitro and in vivo [Soto et al., 1996, 1998]. Proline substitutions were introduced for their propensity to inhibit the β-structure of hydrophobic peptides, and resulted in peptides with a greater capacity to inhibit fibril formation (e.g., RDLPFFDVPID and LPFFD). Furthermore, the pentapeptide reduced the extent of IL-1-positive microglial cells surrounding amyloid deposits in vivo [Sigurdsson et al., 2000]. Hughes et al. [1996] reported that substitution of the two Phe residues located at positions 19 and 20 in the octapeptide (QKLVTTAE) could also inhibit fibril formation by approximately 10-fold, although this was the result of only weak interactions between the octapeptide and monomeric Aβ.

To improve the efficacy of the peptide inhibitors, Soto et al. [1998] developed all D analogs of these peptides, which were just as effective in inhibiting fibril formation, though with increased protease resistance. These so-called "β-sheet breaker peptides" were stable in vivo and exhibited blood-brain barrier permeability, but have yet to progress to human clinical trials [Poduslo et al., 1999; Soto et al., 1998].

An alternative approach to the design of inhibitors of amyloid toxicity has involved the use of a recognition element, which serves to interact specifically with Aβ, linked to a disrupting element, which interferes with normal fibril self-assembly and alters Aβ aggregation pathways. Many of the disrupting elements were based on variants of β-sheet breaker peptides, such as KKKKK or EEEEE [Lowe et al., 2001] or adding amino acids DD [Watanabe et al., 2002]. The anti-aggregant properties of peptide inhibitors, generated using this approach, have been well documented [Ghanta et al., 1996; Lowe et al., 2001; Miyamura et al., 2006; Pallitto et al., 1999].

Ghanta and co-workers designed a prototype peptide inhibitor with a recognition element homologus to the Aβ peptide, 15–25, linked to a lysine hexamer as the disrupting element at the C-terminus [Ghanta et al., 1996]. The peptide modified Aβ aggregation kinetics and protected cells from Aβ-induced toxicity. Furthermore, residues 16–20 in Aβ (KLVFF) were more effective as a recognition element than 15–25, and demonstrated increased efficacy against cytotoxicity [Pallitto et al., 1999]. The scrambled sequence VLFKF was observed to also be just as effective as KLVFF, suggesting that it is the overall hydrophobicity rather than the specific amino acid sequence that is essential. Surprisingly, none of the hybrid inhibitors from the study could prevent Aβ aggregation; rather, they increased aggregate size and changed aggregate morphology. These results strongly suggest that compounds need not prevent aggregation to stop Aβ toxicity. Furthermore, the hybrid inhibitors might work via a mechanism that does not rely on a 1:1 complex with Aβ. Collectively, these studies offer evidence that molecules that can interact with Aβ may interfere with its aggregation and result in inhibition of Aβ-induced pathological readout measures.

N-Methylated Peptides

Peptide N-methylation has also emerged as a powerful tool for inhibition of Aβ and a mechanism to improve peptide half life in vivo [Bodles et al., 2004; Gordon and Meredith, 2003; Gordon et al., 2001; Hughes et al., 2000; Kapurniotu et al., 2002; Kokkoni et al., 2006; Yan et al., 2006]. N-methylation is known to lock the residues into a β conformation [Manavalan and Momany, 1980], generating soluble monomeric β-sheet peptides [Doig et al., 1997]. N-Methylated peptides or "meptides" function by binding to the face of the aggregating peptide through the amide NH groups at the outer edges of the β sheet, effectively blocking intermolecular hydrogen bonding, thus resulting in the prevention of both aggregation and toxicity. Substituents larger than methyl groups [Rijkers et al., 2002] are also effective as inhibitors of amyloidosis.

Hughes and co-workers demonstrated that N-methylated derivatives of $A\beta_{25–35}$ were capable of preventing the aggregation and inhibiting the toxicity of the wild type full-length Aβ peptide [Hughes et al., 2000]. The N-methylated derivatives of $A\beta_{25–35}$ in isolation were soluble and non-toxic because N-methylation blocked hydrogen bonding on the outer edge of the assembling aggregates. Specifically, $A\beta_{25–35}$

with N-methylated Gly33 or Leu34 inhibited fibril assembly entirely and decreased the toxicity of aggregated amyloid.

Gordon and coworkers investigated N-methylated peptides of a region corresponding to 16–22 and later 16–20 of the amyloid "core domain" region to examine their ability to prevent Aβ fibril formation and disassemble preformed fibrils [Gordon et al., 2001, 2002]. Not only did these peptides display high proteolytic resistance, solubility, and membrane permeability [Gordon et al., 2002], they also exhibited a high propensity to form β-structures at the N-methylated site. Cruz and coworkers demonstrated that single N-methyl amino acid-containing peptides similar to 16–20 of Aβ could also reduce the cytotoxicity of $A\beta_{42}$ [Cruz et al., 2004].

The use of specific peptides to inhibit Aβ aggregation and toxicity, although intriguing, has yet to progress beyond in vivo models of amyloidosis. The use of such compounds as molecular markers for the presence of Aβ aggregates has also been suggested as a more viable use for these peptide inhibitors [Esteras-Chopo et al., 2008; Wiesehan et al., 2008]

Small Molecule Inhibitors Based on Amyloid Dyes

Aside from the sequence-based drug design described above, small molecule inhibitors of Aβ aggregation have been modeled based on the histological dyes used to characterize amyloid both in vitro and in vivo. One class of candidates is based on the sulfonated dyes including Congo red, chrysamine G, and thioflavin S. Congo red was reported to inhibit fibrillization and neurotoxicity of Aβ [Fraser et al., 1992; Lorenzo and Yankner, 1994]. Unfortunately, this dye cannot cross the blood-brain barrier and is carcinogenic if given orally, thereby hindering its therapeutic use [Frid et al., 2007]. Due to abundant data supporting the beneficial effects of Congo red interactions with Aβ, the search for a Congo red congener was undertaken. For example, chrysamine G, a smaller and more lipophilic variant of Congo red, has been shown to cross the blood-brain barrier and can block the formation of Aβ fibrils, reduce Aβ toxicity, and increase Aβ proteinase digestion [Klunk et al., 1998].

Aside from Congo red or chrysamine G, other sulfonated dyes can also attenuate the toxic effects of Aβ [Pollack et al., 1995b]. However, those compounds need to adopt a conformation whereby the two sulfonate groups that must be at a similar distance to that displayed in Congo red. This demand implicates a very precise interaction that must occur between the negatively charged sulfonate groups and Aβ for efficacy. Recently, three structural features have been discovered as necessary for an effective and potent inhibitor to Aβ [Reinke and Gestwicki, 2007]. First, a second terminal phenyl group must be present in the inhibitor compound that interacts with Aβ, as it is essential for activity. Ligands that contain simple aromatics failed to be active against Aβ. Second, a hydroxyl substitution on the aromatic end group in the compound is also important for inhibition, as loss of the hydroxl group on the aromatic rings abolished inhibitory activity. Finally, ligands must contain linkers between 8Å and 16Å in distance, and need to be rigid with less than one to two freely rotating carbons. Congo red and chrysamine G are two successful Aβ inhibitors that meet all three of the structural requirements [Masuda et al., 2006; Necula et al., 2007; Porat et al., 2006; Yang et al., 2006]. Of the most significant features is the linker component, as even compounds that conform to the other sub-structural pre-requisites, including aromatic substitutions, failed to inhibit if a short linker is present. A "Goldie-Locks" model was proposed, whereby both the length and flexibility of the linker equally contribute to defining the optimal range and potency of the ligand.

Although dye-based therapeutics have so far not reached clinical trials, these compounds have found application in imaging methodologies [Mathis et al., 2007] and are being investigated in a number of imaging techniques, such as magnetic resonance spectroscopy, position tomography, and single-photon emission computed tomography. The selectivity of these compounds for β-sheet-containing fibers has led to the investigation of these compounds for monitoring the progression of disease in vivo and for distinguishing potential therapeutic effects. At present, the following compounds are in various phases of clinical trials, including [^{11}C]-PiB, a thioflavin analogue, BSB, a Congo red analog, and FSB, a styrylbenzene derivative (www.clinicaltrials.gov). These compounds have a high affinity for amyloid, readily cross the blood-brain barrier, and are presently being used to develop methodologies for AD diagnosis and for monitoring of potential treatments.

Metal Chelators

The role of metal ions, notably Cu^{2+} and Zn^{2+}, in AD and metal chelators as therapeutic agents has been the topic of attention during recent years [Bush, 2003; Caragounis et al., 2007; Doraiswamy and Finefrock, 2004; Gnjec et al., 2002; Raman et al., 2005; White et al., 2006a]. Cu^{2+} decreased Aβ deposits in APP23 transgenic mice [Bayer et al., 2003] and Aβ levels were reduced by a mutant Cu^{2+} transporter [Phinney et al., 2003]. Additionally, overexpression of human Aβ peptides in transgenic mice decreased brain Cu^{2+}

[Maynard et al., 2002]. APP knock-out mice also displayed elevated levels of Cu^{2+} [White et al., 1999]. Although many reports have ascribed a major role for metal ions in AD, a substantial amount of research has also pointed to the deleterious effects of metal ions in the development of AD. While these metals are essential in most biological reactions, their excessive accumulation can be cytotoxic, as an imbalance in metal homeostasis can result in a vast range of cellular disturbances typified by oxidative stress and elevated levels of superoxide or free radical production. Precipitation and aggregation of Aβ peptides to form senile plaques and NFTs have been documented [Fisher and Naughton, 2005].

The biochemical mechanisms by which metal ligands affect Aβ metabolism has been of great interest. The anti-malarial 8HQ (8-hydroxyquinoline) derivative 5-chloro-7-iodo-8-hydroxyquinoline (CQ; clioquinol), is a transition metal ion chelator [Di Vaira et al., 2004; Lane et al., 1960]. CQ can dissolve plaque deposits of AD brain tissue in vitro [Bush, 2003], and decrease deposits in animal AD models. [Bush, 2003; Cherny et al., 2001]. CQ-metal complexes can also up-regulate matrix metalloprotease (MMP) activity in vitro by activating phosphoinositide 3-kinase (PI3K) and c-jun N-terminal kinase (JNK). Enhanced MMP activity increased degradation of secreted Aβ peptide [White et al., 2006b].

Caragounis et al. [2007] studied different classes of metal ligands that increase cellular metal levels, showing that Cu^{2+} and Zn^{2+} resulted in considerable loss of secreted Aβ. The metal ligands that reduced Aβ levels included 8HQ (8-hydroxyquinoline) and phenanthroline derivatives, as well as the sulfur compound PDTC (pyrrolidine dithiocarbamate). The authors speculated that the inhibitory affect was due to a higher lipid solubility of the ligands and their ability to enhance metal uptake. However, it was also possible that the ligands could effectively inhibit Aβ levels without altering cellular metal homeostasis [Treiber et al., 2004; White et al., 2006a]. These results suggest that a host of metal ligands can have analogous results on Aβ turnover, with a number of lipid-soluble ligands significantly decreasing extracellular levels of Aβ once complexed to Cu^{2+} or Zn^{2+}. This reduction could be attained through metal-dependent activation of JNK and upregulation of MMP activity, and, as a result, an up-regulation of metalloprotease activity and consequent loss of secreted Aβ.

CQ administration to AD patients dramatically reduced progression of cognitive decline and coincided with a decrease in plasma $A\beta_{42}$ levels [Ritchie et al., 2003]. While CQ was of interest as a potential drug [Bush, 2003; Ritchie et al., 2003; Yassin et al., 2000] for AD treatment, its evaluation has been discontinued due to deleterious side effects in Japanese patients and efforts have been focused on novel, non-toxic congeners of CQ. A third-generation clioquinol, PTB2, has finished a small Phase IIa clinical trial in early AD patients (www.pranabio.com). PBT2 had a good safety and tolerability profile, and at a high dose reduced cerebrospinal fluid $A\beta_{42}$ levels compared to placebo. These results supported the in vivo findings in a transgenic mouse model of AD, where PBT2 reduced toxic oligomers of Aβ, reversed Aβ-induced loss of neurotransmission and improved cognition (www.pranabio.com). The ultimate efficacy and safety profile of this class of compounds will await further human clinical trial data.

Polyphenols

Polyphenols are a large group of synthetic and naturally occurring small molecules that are composed of one or more aromatic phenolic rings and are divided into three main groups, including phenolic acids, flavonoids, and non-flavonoid polyphenols [Ramassamy, 2006]. Experimental and epidemiological evidence suggests that natural polyphenolic compounds, such as those found in teas, berries, fruits, spices, and plants, have antioxidative, anti-inflammatory, and anti-aggregant properties. Given the multifaceted nature of Aβ-related neurotoxicity, much work has been done to examine both the direct interaction of polyphenols on Aβ fibrillogenesis and on secondary effects, such as Aβ-induced pro-apoptotic mechanisms. As over 8,000 polyphenolic compounds are known, this discussion will focus on the interaction between amyloid peptides and three well-characterized naturally occurring phytochemicals; curcumin, (−)epigallocathechin-3-gallate, and Ginkgo biloba (Fig. 2).

Curcumin

Epidemiological studies indicating a significantly lower prevalence of AD in the Asian Indian population compared to the United States suggest the possibility of a dietary correlation [Ganguli et al., 2000; Ramassamy, 2006]. The common use of tumeric as a curry spice has pointed to its main constituent, curcumin, as a possible mediator of anti-Aβ effects. Indeed, curcumin and related compounds like calebin-A, dimethoxycurcumin, and bidemethoxycurcumin, inhibit fibril formation and extension and promote destabilization of pre-aggregated Aβ peptides [Kim et al., 2005; Ono et al., 2004; Park and Kim, 2002; Yang et al., 2005]. Examination of the structure-activity function of curcumin has identified three important molecular features, including a hydroxyl substitution on the aromatic end group, a narrow linker length between 8–16 Å, and the presence

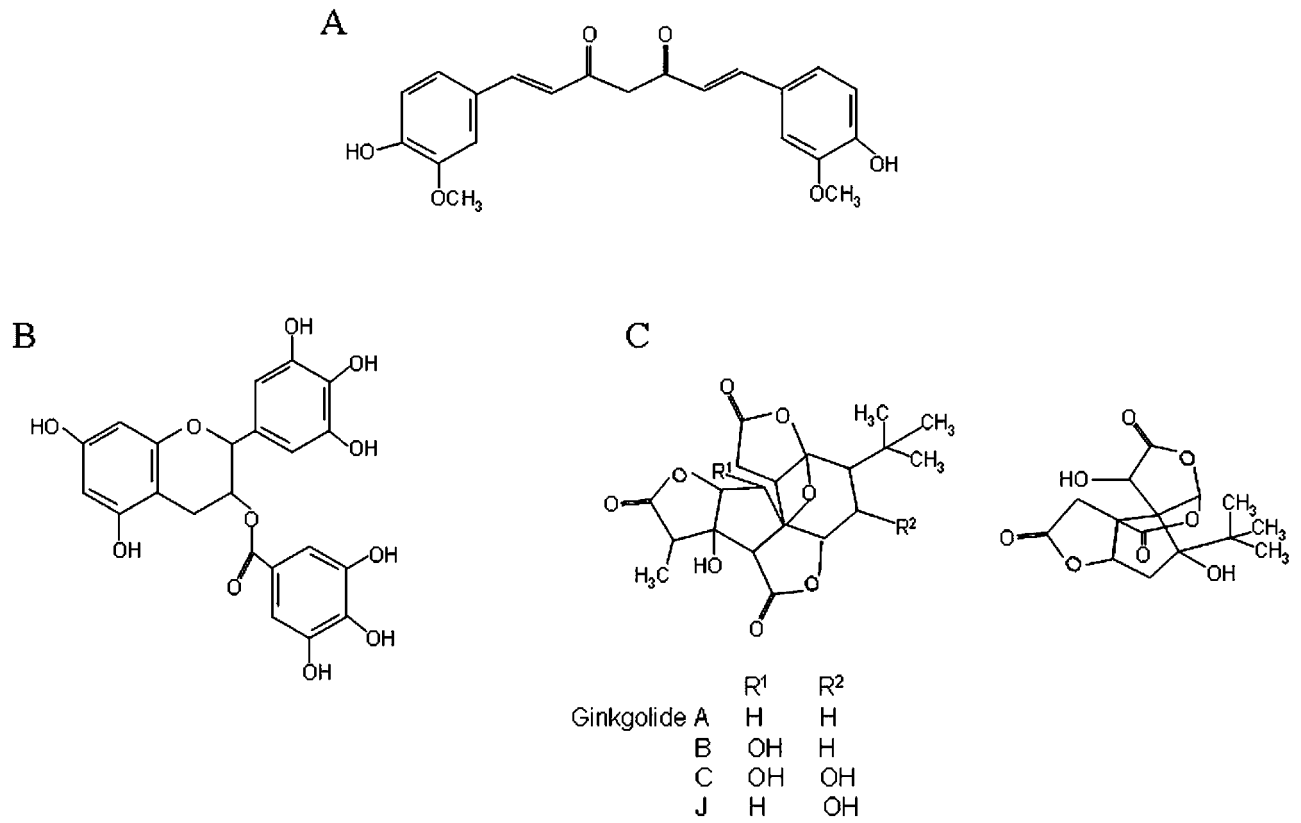

Fig. 2. Schematic of the chemical structure of curcumin (**A**), (−)epigallocatechin-3-gallate (**B**), Ginkgolides (**C**, left) and bilobalides (**C**, right).

of a second terminal phenyl group that determines its anti-aggregant properties [Reinke and Gestwicki, 2007]. In vitro, curcumin protects against Aβ-induced death of PC-12, SH-SY5Y neuroblastoma, and human umbilical vein endothelial cells, via anti-oxidant actions [Baum and Ng, 2004; Kim et al., 2001; Park and Kim, 2002; Yang et al., 2005]. Not surprisingly, intravenously-injected curcumin binds to parenchymal and vascular amyloid deposits in the brains of $TgAPP_{swe}$/PS1 and Tg2576 mice [Garcia-Alloza et al., 2007; Yang et al., 2005]. Additionally, promising anti-amyloid effects have also been noted in TgAPP mice fed with varying doses of curcumin. For instance, Lim et al. [2001] reported decreased concentrations of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ peptides, an amelioration of plaque burden, decreased amounts of oxidized proteins, and lower interleukin-1β (IL-1β) levels in the brains of 10-month-old $TgAPP_{swe}$ mice fed curcumin for 6 months. Similar effects were also noted in 17-month-old Tg2576 mice, suggesting the effectiveness of curcumin in clearing pre-existing plaques in vivo [Yang et al., 2005]. In addition, administration of curcumin to adult rats injected with $A\beta_{42}$ showed improved performance on the Morris water maze and an 80% reduction of plaque load throughout the brain, which correlated with a reversal of Aβ-induced decreases in post-synaptic density 95 protein levels [Frautschy et al., 2001]. Collectively, these findings suggest that the neuroprotective capabilities of curcumin result from its ability to directly alter the kinetics of Aβ fibrillization, as well as its antioxidative and anti-inflammatory properties. Curcumin is currently the focus of two clinical trials in China and the United States for the evaluation of its effectiveness in AD patients.

(−)-Epigallocatechin-3-Gallate

Catechins are a family of plant-derived flavan-3-ols comprised of (−)-epicatechin, (−)-epigallocatechin, (−)-depicatechin-3-gallate, and (−)-epigallocatechin-3-gallate (EGCG). EGCG is the major polyphenolic component of green tea and has been implicated in the prevention of age-related neurodegenerative disorders such as AD. A variety of experimental in vitro cell culture paradigms have shown micromolar concentrations of EGCG to be protective against Aβ-induced cell death [Bastianetto et al., 2006; Kim et al., 2007; Levites et al., 2003]. Further, i.p. and intracerebroventricular administration of EGCG to Tg2576 mice decreases soluble and insoluble levels of $A\beta_{40}$ and $A\beta_{42}$ and

reduces plaque load by 40–50% across the hippocampus and cortex of treated animals [Rezai-Zadeh et al., 2005]. A variety of mechanisms, including anti-aggregation, anti-inflammatory, antioxidative, and iron-chelating capabilities have been proposed to mediate these neuroprotective effects [Bastianetto et al., 2006; Kim et al., 2007; Levites et al., 2003; Obregon et al., 2006; Rezai-Zadeh et al., 2005; Reznichenko et al., 2006]. For instance, EGCG treatment induced an upregulation in pro-survival mitogen activated protein kinase (MAPK) phosphatase-1 levels and antagonized Aβ/IL-1-induced p38MAPK and JNK phosphorylation [Kim et al., 2007].

EGCG also appears to inhibit Aβ toxicity by promoting non-amyloidogenic APP processing. Treatment of human SH-SY5Y neuroblastoma and $N2a_{swe}$ cells with EGCG induced a dose- and time-dependent increase in the levels of sAPPα and secreted α-C-terminal fragment (CTF), without altering holo-APP expression [Levites et al., 2003; Rezai-Zadeh et al., 2005]. In addition, EGCG administration increased the expression of two putative α-secretases, TACE and ADAM-10, correlating with increased levels of αCTF and sAPPα and decreased concentrations of $Aβ_{40}$ and $Aβ_{42}$. This effect was abolished in the presence of the α-secretase inhibitor, Ro31-9790, or following ADAM-10 siRNA knockdown [Levites et al., 2003; Obregon et al., 2006; Rezai-Zadeh et al., 2005]. Furthermore, EGCG dose-dependently inhibits BACE1 activity in vitro [Jeon et al., 2003]. Collectively, these results indicate that the neuroprotective effects of EGCG and other green tea catechins are due, at least in part, to the ability of EGCG to directly modulate APP processing and to counteract the pro-inflammatory and pro-oxidative cellular environment induced by Aβ exposure.

Gingko Biloba

Gingko biloba extracts have been used for centuries in traditional Chinese medicine for their antioxidant and anti-apoptotic effects. The standardized concentrated extract, EGb-761, contains a mixture of 27% flavonols (e.g., quercetin, kaempferol, and isorhamnetin) and 6–7% terpene lactones (e.g., bilobalide and gingkolides A, B, and C) [Augustin et al., 2008; Ramassamy, 2006; Vitolo et al., 2007]. Both EGb-761 and its constituents have been investigated for their neuroprotective properties against Aβ-induced toxicity. Treatment with EGb-761 and its flavonoid fraction CP205 rescues N2a, PC-12, and primary mixed hippocampal cell cultures from Aβ-stimulated apoptosis by counteracting elevations in reactive oxygen species, and preventing NF-κB activation [Bastianetto et al., 2000; Longpre et al., 2006; Yao et al., 2001]. Such anti-inflammatory and antioxidative properties might account in part for the protective effects of ginkgolides A, B, and J in reducing $Aβ_{42}$-induced decreases in synaptophysin levels and restoring long-term potentiation in hippocampal slice cultures [Bate et al., 2008; Vitolo et al., 2007]. Recent in vivo findings have shown that treatment with Gingko biloba or EGb-761 improves motor performance and rescues spatial memory impairments in APP transgenic *Caenorhabditis elegans* worms and Tg2576 mice [Stackman et al., 2003; Tchantchou et al., 2007; Wu et al., 2006]. In most cases, EGb-761 treatment decreased the level of 20–28 kDa Aβ oligomers, without affecting APP transgene expression [Tchantchou et al., 2007; Wu et al., 2006].

Recently, it has also been reported that BACE1 mRNA levels and activity were not altered in EGb-761-treated N2a cells, nor in Tg2576 mice receiving EGb-761 over a 4-week period [Augustin et al., 2008]. Furthermore, EGb-761 treatment did not affect levels of ADAM10 or ADAM17 in rats given EGb-761, despite an upregulation of sAPPα expression [Colciaghi et al., 2004]. These data suggest that EGb-761 inhibits Aβ fiber growth directly, rather than by acting upon APP secretase activity. This hypothesis is supported by in vitro findings that EGb 761 and CP205 were able to inhibit oligomeric formation of Aβ-derived diffusible soluble ligands (ADDLs) and prevent fibril formation at low doses [Chromy et al., 2003; Longpre et al., 2006; Yao et al., 2001]. Gingko in combination with curcumin is presently in a Phase II trial in Hong Kong, where the safety and tolerability of these compounds are being evaluated in AD patients.

Glycosaminoglycan mimetics

Glycosaminoglycans (GAGs) are unbranched linear polymers of repeated disaccharide units that attach covalently to a protein core to form proteoglycans. Non-sulfated GAGs include hyaluronic acid, while sulfated GAGs are comprised of chondroitin, keratan, dermatan, and heparan sulfates [Diaz-Nido et al., 2002; van Horssen et al., 2003]. The hypersulfated form of heparan sulfate is referred to as heparin [Diaz-Nido et al., 2002]. Chondroitin sulfate, heparan sulfate, and hyaluronic acid have all been identified in the developing and mature rodent brain and may be involved in cell attachment and neurite outgrowth [Oohira et al., 2000].

An association between GAGs/proteoglycans and AD has been suggested since the 1850s, when amyloid deposits in kidney, liver, and spleen were found to contain carbohydrates [Kisilevsky et al., 2007]. Confirmation of a proteoglycan accumulation in AD brain lesions was provided by Snow and colleagues, who

demonstrated the presence of sulfated GAGs, predominantly that of heparan and chondroitin sulfate, in neuritic plaques, vascular amyloid deposits, and neurofibrillary tangles [Snow et al., 1988, 1990]. A role for sulfated GAGs and related proteoglycans in the formation and/or promotion of Aβ deposition has also been suggested from in vitro and in vivo experiments. Perlecan, an extracellular matrix-associated heparan sulfate glycoprotein, binds directly to both $A\beta_{40}$ and $A\beta_{42}$ and dose-dependently increases the amount, rate, and stability of fibril formation [Castillo et al., 1997]. Furthermore, binding of heparan sulfate and chondroitin sulfate proteoglycans to fibrillar Aβ, prevented its proteolytic degradation, an effect that is blocked in the presence of free GAG chains [Gupta-Bansal et al., 1995]. Co-infusion of $A\beta_{40}$ plus heparan sulfate proteoglycan into the hippocampus of adult rats significantly increased Aβ plaque deposition, compared to rats injected with $A\beta_{40}$ alone [Snow et al., 1994]. A direct role for GAGs in Aβ fibril formation has been further suggested by reports that heparin, chondroitin sulfate A, keratan sulfate, dermatan sulfate, dextran sulfate, and heparan sulfate are all able to accelerate fibril nucleation and extension [Castillo et al., 1999; McLaurin et al., 1999].

Given that fibrillar Aβ is now considered to be the least toxic of the amyloid peptide conformations, it is perhaps not surprising that GAG-Aβ interactions are neuroprotective. Treatment of rat primary hippocampal cultures with chondroitin sulfate or heparan sulfate attenuated Aβ-induced neurite fragmentation and cellular toxicity [Woods et al., 1995]. Administration of the low molecular weight heparin, enoxaparin, also dose-dependently inhibited Aβ-induced toxicity in PC-12 neuroblastoma cells [Bergamaschini et al., 2004]. Similarly, highly sulfated GAGs, such as carrageenan-τ pentosan sulfate and heparin sulfate protect against decreased cell viability caused by Aβ in PC-12 cells, while less sulfated GAGs were less effective [Pollack et al., 1995a]. The importance of sulfate group number and distribution on the GAG backbone for the effectiveness of its interaction with Aβ [Fraser et al., 1992; Leveugle et al., 1994], was confirmed by reports that heparin-induced increases in Aβ fibril formation were impaired following removal of O- and N-sulfates and were completely lost upon removal of all sulfate moieties from heparin [Castillo et al., 1998, 1999].

Further examination of the structure-activity relationships mediating GAG-Aβ binding have led to the proposal that a cluster of basic amino acids in the N-terminal region of the Aβ peptide (residues 13–16) affect its secondary structure, thereby influencing the peptide interaction with GAGs [McLaurin and Fraser, 2000]. In addition, fluorescent spectroscopy and electron microscopy experiments have demonstrated that sulfated mono- and disaccharides derived from chondroitin sulfate bind Aβ directly, competing with intact chondroitin sulfate and heparin for Aβ binding [Fraser et al., 2001]. Furthermore, chondroitin sulfate-derived disaccharides were sufficient to induce lateral fiber aggregation and conversion of protofibrils into mature amyloid fibers [Fraser et al., 2001]. These results suggested that the development of small, sulfated compounds that cross the blood-brain barrier and mimic GAG-Aβ binding might be therapeutically useful for sequestering toxic Aβ species.

As such, some GAG-based therapies have been based on the use of low molecular weight heparin derivatives, many of which are currently used in the treatment of venous thromboembolism. Zhu et al. [2001] reported that pre-treatment with either enoxaparin or dalteparin arrested the progression of inflammation-associated amyloid induction by blocking β-pleated sheet formation. Similarly, Bergamaschini et al. [2004] demonstrated that chronic (e.g., 6 months) peripheral administration of enoxaparin to TgAPP23 mice significantly reduced cortical plaque load, decreased total $A\beta_{40}$ levels, and attenuated the number of plaque-associated astrocytes. Interestingly, treatment of rats with certoparin or its derivative C6, either before or after intra-amygdaloid infusion of Aβ, blocked Aβ-associated changes to intracellular tau and reduced astrogliosis, without altering Aβ aggregation [Walzer et al., 2002]. Similar findings were also reported by Dudas et al. [2002] using the C3 derivative, which protects against Aβ-induced increases in tau-2-immunoreactivity in hippocampal neurons. Another set of GAG-based anti-amyloidogenic compounds has been designed according to their ionic properties. Kisilevsky et al. [1995] reported that oral and ip administration of poly(vinylsufonate sodium salt), a small-molecule anionic sulfonate, inhibited splenic Aβ deposition in a mouse model of inflammation-associated amyloid induction, under both acute and chronic conditions. A different GAG mimetic, 3-amino-1-propanesulfonic acid (Tramiprosate), binds soluble $A\beta_{40}$ and $A\beta_{42}$ peptides and maintains them in a random-coil conformation [Gervais et al., 2007]. Treatment of rat neuronal cell cultures or SH-SY5Y neuroblastoma cells with tramiprosate significantly attenuated $A\beta_{42}$-induced toxicity. Furthermore, administration of tramiprosate to TgCRND8 mice reduced compact plaque load and decreased plasma levels, as well as soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ concentrations in the brains of treated mice, compared to control [Gervais et al., 2007]. Although the behavioral effects of this compound have not been reported, tramiprosate has been tested in AD and cerebral amyloid angiopathy

[Aisen et al., 2006; Greenberg et al., 2006]. Phase III clinical trials in AD did not reach clinical significance in the cognitive outcomes, but due to the good safety and tolerability trials, this compound will be marketed as a neutraceutical (www.bellushealth.com).

Lipid-Based Small Molecule Inhibitors

The interaction of Aβ with lipids and cellular membranes has been known for some time, although the outcome of this interaction is still controversial. Lipids have been implicated as potential accelerators of Aβ fibrillogenesis and the target of Aβ-induced toxicity thus propagating pathogenesis. Aβ deposition is initiated in a plasma membrane–bound form resulting in diffuse plaque formation [Yamaguchi et al., 2000]. In addition, a "seeding" form of Aβ in culture is membrane associated and dependent on cholesterol levels [Mizuno et al., 1999]. In vitro studies on isolated hippocampal membranes have shown that Aβ has an enhanced disordering effect on AD membranes as compared to age-matched controls [Eckert et al., 2000]. These studies, in conjunction with the enhanced Aβ association with the cell surface [Burdick et al., 1997; Yang et al., 1998], have led to speculation that Aβ-cell membrane interactions are important for the development of amyloid deposits. Understanding Aβ interactions with specific lipid families may lead to the development of lipid mimetics that could inhibit membrane interactions and subsequent Aβ toxicity.

The interaction of Aβ with various classes of lipids, including glycolipids, cholesterol, and phospholipids, has shown that each lipid family is important and led to the investigation of a number of potential treatment strategies. Glycolipids, particularly gangliosides, either inhibit Aβ aggregation by stabilization of a novel α/β conformation [McLaurin et al., 1998] or promote Aβ aggregation [Choo-Smith et al., 1997; Kakio et al., 2002], as demonstrated by the isolation of a novel ganglioside-bound Aβ species from AD brain [Yanagisawa and Ihara, 1998; Yanagisawa et al., 1995].

In contrast, acidic phospholipids induce β-structured aggregates and fibers [McLaurin and Chakrabartty, 1996; Terzi et al., 1995; Waschuk et al., 2001]. PEGylated phospholipid nanomicelles interact with Aβ thereby mitigating β-structural transitions, aggregation, and subsequent neurotoxicity [Pai et al., 2006]. The interaction of Aβ with phosphatidylinositol produced a dramatic increase in fibrillogenesis that was inhibited by introduction of phosphate groups present in the other phosphatidylinositol family members of lipids [McLaurin et al., 1998]. *myo*-Inositol, the head group of phosphatidylinositol, inhibited Aβ aggregation and Aβ-induced toxicity [McLaurin et al., 1998]. Since *myo*-inositol is a native constituent of the CNS, the effects of the various inositol stereoisomers on Aβ-aggregation and toxicity were examined [McLaurin et al., 2000; Nitz et al., 2008] and showed a stereospecific interaction of inositol with Aβ that required all hydroxyl groups to be in an equatorial position for maximal activity, the *scyllo*-inositol isomer [McLaurin et al., 2000]. *scyllo*-Inositol administration to TgCRND8 mice prevented Aβ-induced cognitive deficits, plaque formation, synaptotoxicity, and early death [McLaurin et al., 2006]. These effects were seen in both prophylactic and treatment studies. Peripheral administration of *scyllo*-inositol also prevented memory deficits in an acute rat model of Aβ-toxicity [Townsend et al., 2006]. These *in vivo* effects resulted from the inhibition of Aβ oligomer induced toxicity in the rat, and the prevention of high molecular weight oligomer formation in the Tg mouse model of AD. *scyllo*-Inositol also prevents Aβ oligomer-induced synaptotoxicity in a mouse model of AD [Shankar et al., 2007]. The benefit of *scyllo*-inositol may result from the high CNS bioavailability due to the constitutive activity of the inositol transporters at the blood brain barrier [Fenili et al., 2007].

scyllo-Inositol had favorable pharmacokinetics, safety, and tolerability in a Phase I clinical trial (www.transitiontherapeutics.com) with Phase II clinical trials initiated in mild to moderate AD patients in December 2007 (www.clinicaltrials.gov).

CONCLUSION

Although small molecule anti-aggregant inhibitors have not shown efficacy in clinical trials, such strategies are approaching proof of concept. Clinical trials utilizing active immunization have suggested that removal of Aβ from the CNS is possible, but effects on cognitive function, while suggestive, are unclear. Ongoing clinical trials may help to elucidate the full potential of small molecule therapies to improve the quality of life of patients with AD.

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