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Amyloid β-Protein Assembly as a Therapeutic Target of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD), the most common neurodegenerative disorder in the aged, is characterized by the cerebral deposition of fibrils formed by the amyloid β-protein (Aβ), a 40–42 amino acid peptide. The folding of Aβ into neurotoxic oligomeric, protofibrillar, and fibrillar assemblies is hypothesized to be the key pathologic event in AD. Aβ is formed through cleavage of the Aβ precursor protein by two endoproteinases, β-secretase and γ-secretase, that cleave the Aβ N-terminus and C-terminus, respectively. These facts support the relevance of therapeutic strategies targeting Aβ production, assembly, clearance, and neurotoxicity. Currently, no disease-modifying therapeutic agents are available for AD patients. Instead, existing therapeutics provide only modest symptomatic benefits for a limited time. We summarize here recent efforts to produce therapeutic drugs targeting Aβ assembly. A number of approaches are being used in these efforts, including immunological, nutraceutical, and more classical medicinal chemical (peptidic inhibitors, carbohydrate-containing compounds, polyamines, "drug-like" compounds, chaperones, metal chelators, and osmolytes), and many of these have progressed to phase III clinical trails. We also discuss briefly a number of less mature, but intriguing, strategies that have therapeutic potential. Although initial trials of some disease-modifying agents have failed, we argue that substantial cause for optimism exists.

INTRODUCTION

Alzheimer's disease (AD) is the most common late-life neurodegenerative disorder [1], affecting an estimated 5.2 million in the U.S. and >27 million worldwide [2]. These case

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numbers are expected to triple or quadruple by 2050 [2]. If this should occur, the economic cost of AD patient care, now estimated at >$100 billion per year [3], will bankrupt the U.S. health care system [4]. Unfortunately, no disease-modifying therapies now exist. Coupled with the unquantifiable misery suffered by AD patients and their families around the world, the need for ameliorative and curative drugs is especially acute.

The most prominent current working hypothesis of AD pathogenesis posits that the amyloid β-protein (Aβ), an ~4,300–4,500 molecular weight peptide, is the proximate neurotoxic agent (for a recent review, see Roychaudhuri *et al.* [5]). Neurotoxicity is thought to result from the self-association of Aβ into oligomeric and higher order assemblies. Aβ itself is produced through the sequential action of two endoproteinases, β-secretase and γ-secretase, that cleave the Aβ N-terminus and C-terminus, respectively, from the larger Aβ precursor protein (APP) [1]. These facts support the relevance and attractiveness of two predominant strategies for the development of AD therapeutics: (1) blocking Aβ production; and (2) blocking Aβ self-assembly.

In this review, we focus on efforts to develop therapeutic agents targeting Aβ assembly (Table 1, Fig. 1). This process is surprisingly complex [5], which may explain why no one agent or class of agents yet has emerged as an obvious and favored choice for drug development. In fact, in addition to classical drug-like compounds, immunoglobulins, proteins, peptides, carbohydrate-containing compounds, lipids, nucleic acids, polyamines, osmolytes, chelators, polyphenols, vitamins, and other agents all are being studied. Such a broadly based search for efficacious compounds is especially valuable in light of initial, and well-publicized, failures of clinical trials representing diverse classes of therapeutic agents. In the sections that follow, we seek to provide the reader with a comprehensive, but necessarily brief, introduction to each of the better-developed approaches extant, as well as some insight into nascent but exciting new therapeutic strategies.

THERAPEUTIC APPROACHES

1. Immunotherapy

Immunotherapy involves the activation of cell-mediated or humoral (antibody) immune responses to eliminate noxious agents from the body. In AD, as discussed above, the “noxious agent” appears to be Aβ. Immunological approaches to AD therapy thus have focused on humoral immune responses specific for Aβ. Two classes of immunotherapeutic intervention have been explored: active and passive immunization. In the former class, various types of Aβ immunogens are used to elicit endogenous Aβ-specific antibody production. In the latter class, antibodies are produced exogenously, e.g., through monoclonal antibody methods, and then administered passively to the affected host.

Several mechanisms of action are thought to underlie the effects of Aβ-specific antibodies on Aβ assembly and amyloid plaques. One mechanism suggests that antibodies cross the blood-brain barrier (BBB), enter the brain, bind to Aβ in plaques, and target them for phagocytosis [6]. Active immunization trials in transgenic (Tg) mouse models of AD support this hypothesis. For example, Schenk *et al.* demonstrated that active immunization with Aβ42 prevented plaque formation in young mice and significantly reduced the plaque

load in older mice [7]. The remaining Aβ plaques in these immunized mice exhibited significant amounts of bound antibodies. Major histocompatibility complex (MHC) II-expressing cells, thought to be activated microglia and monocytes, also were associated with these remaining plaques [7]. A second mechanism is direct antibody-mediated dissociation of Aβ fibrils and aggregates, which produces soluble forms of Aβ that can be eliminated more readily from the body. Such fibril disruption has been observed *in vitro*, where Aβ-specific antibodies can prevent or reverse fibril formation [8–10]. A third mechanism suggests that antibodies alter brain Aβ levels by affecting the equilibrium between brain and plasma Aβ. This "peripheral sink" theory posits that circulating Aβ-specific antibodies bind to and eliminate extra-cranial Aβ. To reestablish the intrinsic equilibrium between intra-and extra-cranial Aβ, insoluble Aβ in plaques is consumed and peripheral Aβ levels again rise. Antibodies thus need not cross the BBB to cause plaque elimination [11, 12]. Holtzmann *et al.* showed that peripheral administration of a monoclonal antibody (m266) specific for the central region of Aβ to PDAPP (platelet-derived growth factor promoter-expressing amyloid precursor protein) mice caused a 1,000-fold increase in plasma Aβ levels [11, 12]. Furthermore, the m266 antibody markedly reduced Aβ deposits in the mice, despite the inability of the antibodies to bind directly to these deposits.

Other mechanisms also may operate. For example, antibodies may neutralize neurotoxic Aβ assemblies by direct binding, a process independent of antigen clearance mechanisms. Antibody-independent mechanisms also may occur, as suggested by studies of innate immune responses mediated by macrophages and other phagocytic cells [13], responses that do not depend on the action of opsonizing antibodies [14].

a. Active Immunization—The first active immunization trial involved the administration of the immunogen AN1792, which is aggregated Aβ, along with a QS21 (*Quillaja Saponaria* 21) surface active saponin adjuvant. Immunization with AN1792 decreased plaque load in Tg mice that usually form plaques similar to those found in AD patients [7] and ameliorated behavioral impairments [15, 16]. Based on this evidence, phase I clinical trials were started on 80 patients with mild-to-moderate AD. The patients were immunized with AN1792 in adjuvant four times over a six-month period. Overall, the results from this trial showed promising results, including the induction of an Aβ-specific antibody response and delayed cognitive decline, based on the Disability Assessment Scale for Dementia (DAD). These results supported the initiation of a phase II trial [17]. However, during the phase II trial, one of the patients from the phase I trial developed subacute encephalitis and died from non-CNS related causes [18]. Subsequently, 18 of the 300 patients in the phase II trial developed subacute encephalitis, resulting in the early termination of the study [19]. Other Aβ vaccination protocols are under development. Elan/Wyeth started phase I testing of ACC-001, a vaccine that comprises the N-terminal seven amino acids of Aβ attached to a mutated diphtheria toxin carrier protein, CRM 197 (cross-reacting material 197). However, recently (April 2008), experimental testing of this vaccine was suspended after one patient developed skin lesions [20, 21]. It was unclear whether this patient was a member of the placebo or vaccine-treated group. Two other phase I vaccination trials are underway, CAD-106 from Novartis [22] and V-950 from Merck [23]. CAD-106 consists of

Immunodrug™ carrier Qb coupled to an undisclosed fragment of Aβ. The nature of the V-950 immunogen is unknown.

b. Passive Immunization

i. Bapineuzumab (AAB-001): Bapineuzumab, from Elan/Wyeth, is a humanized monoclonal antibody (half-human, half-mouse antibody produced in a mammalian cell culture system) specific for the N-terminus of Aβ. Pre-clinical assays showed that the mouse version of this antibody had affinity to both soluble and aggregated Aβ. Bapineuzumab currently is being tested in a phase III trial on patients with mild-to-moderate AD at 200 study sites in North America [24] and >150 study sites in 20 countries outside the U.S. [25, 26].

Results from the phase II clinical trial reported recently at the International Conference on Alzheimer's Disease (ICAD) were disappointing [27]. In the double-blind, randomized, placebo-controlled trial (DBRPCT), 234 patients received one of four doses of Bapineuzumab (0.15 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg) or placebo by intravenous (i.v.) administration every 13 weeks for 18 months. No significant differences were observed in the overall study population in the clinical efficacy measures Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) or DAD. Furthermore, Bapineuzumab did not exhibit a clear does-response profile typical of most pharmaceutical agents. Nevertheless, *post hoc* apolipoprotein ε4 (APOE4) haplotype analysis revealed promising statistical results. In non-APOE4 subjects, the Bapineuzumab-treated group showed significant improvement compared to the placebo group by several metrics, including ADAS-Cog (p=0.026), Neuropsychological Test Battery (NTB; p=0.006), Clinical Dementia Rating Sum of Boxes (CDR-SB; p=0.04), and decrease in brain volume as determined by the Brain Boundary Shift Integral (BBSI; p=0.004). Additionally, trends toward improved DAD and smaller increases in ventricular volume were noted in the Bapineuzumab-versus placebo-treated non-APOE4 subjects. In APOE4 subjects, in contrast, no statistically significant changes were observed in any of the cognitive or functional endpoints, although favorable trends were noted.

Bapineuzumab treatment exhibited a generally mild and transient side effect profile, except for the appearance of vasogenic edema that presented in 12 treatment patients receiving higher Bapineuzumab doses. The incidence of vasogenic edema, which correlated positively with APOE4 allele status, resolved after cessation of treatment.

Despite the lack of a clear dose-response, the potentially serious side effect of vasogenic edema in the APOE4 patients, and the difficulty in establishing efficacy in the overall population group, Bapineuzumab may have promise for non-APOE4 AD patients. It is hoped that results from the ongoing phase III trial of Bapineuzumab, in which a much larger number of participants exists than in the phase II trial, will provide an answer.

ii. LY2062430: LY2062430 is passive immunization therapy currently in phase II trials [28] that was developed by Eli Lilly and uses a human monoclonal antibody targeting the central domain of Aβ. Preclinical studies with the mouse analogue of this antibody, m266, demonstrated decreased plaque formation [11] and increased behavioral performance [29,

30]. Promising results from the phase II clinical trial recently were reported at ICAD. In this 12-week study, 52 patients with mild-to-moderate AD were given placebo or LY2062430 using dosing schedules of 100 mg or 400 mg every week or 100 mg or 400 mg every month. LY2062430 was well tolerated and no evidence was observed of inflammation, bleeding, or other cerebral side effects up to the tested 400 mg weekly infusions. Importantly, i.v.-administered LY2062430 increased plasma and cerebrospinal fluid (CSF) concentrations of Aβ, suggesting that the treatment facilitated plaque elimination.

iii. Intravenous Immunoglobulin (IVIg): Intravenous immunoglobulin (IVIg) is an allogeneic polyclonal antibody treatment that putatively reduces Aβ levels in the brain and improves cognitive abilities in AD patients [31]. IVIg already was an FDA-approved therapy for several autoimmune and immunodeficiency diseases, yet its efficacy in AD has not been established. Human IVIg strongly inhibits fibril formation [32] and dissociates preformed Aβ fibrils *in vitro* [33]. IVIg also almost completely abrogates Aβ-induced neurotoxicity in rat hippocampal cells [32]. The mechanism of IVIg action may include increased cellular resistance to Aβ neurotoxicity and enhanced microglial phagocytosis of Aβ deposits [33].

A pilot clinical study of five AD patients suggested that IVIg administered over 6 months resulted in significant decreases in total Aβ levels in CSF and serum, with significant cognitive improvement as measured using the ADAS-Cog [34]. A follow-up open label study was conducted on 8 mildly affected AD patients treated with 2 g IVIg/kg for 6 months, followed by a 3-month washout period, and a final treatment regimen of 9 months [31]. CSF Aβ concentrations decreased during IVIg administration but returned to pre-treatment levels after washout. Interestingly, mini-mental state examination (MMSE) scores increased an average of 2.5 points after the initial treatment, but returned to baseline after washout and remained stable during the second phase of treatment [31]. No significant adverse events were noted in this study, suggesting that IVIg treatment is well tolerated. Currently, IVIg is in phase II testing in a 6-month long DBRPCT [35].

iv. Other Trails: Two other passive immunization trials currently are in phase I testing, RN-1219, a humanized Aβ-specific monoclonal antibody, and R-1450 [36], a monoclonal antibody developed from a fully synthetic human combinatorial antibody library (HuCAL) [37].

2. Nutraceuticals

Nutraceuticals are natural products or extracts therefrom that display medicinal or health benefits, independent of their nutritive properties. In the majority of cases, claims of health benefits from these compounds are unsupported by rigorous scientific studies. However, an increasing body of pre-clinical research, and certain clinical studies, suggest that some nutraceuticals may be of value as AD therapeutic agents.

a. Grape Seed Extracts—Recent epidemiological studies suggest that consumption of moderate amounts of red wine may protect against the development of AD [38–42]. Red wine is known to contain a broad range of polyphenols derived from the skin and seeds of grapes [43–47]. Several reports on the effects of these polyphenols suggest why

consumption of red wine correlates with decreased AD risk. Wine-related polyphenols are potent anti-oxidants. Oxidation reactions have been postulated to be important pathologic processes in AD. Polyphenols thus may protect neurons from oxidative injury by scavenging reactive oxygen species, including hydroxyl or superoxide radicals [48, 49]. However, a number of reports show that wine-related polyphenols also inhibit Aβ fibril formation, fibril elongation, and dissociate pre-formed Aβ fibrils *in vitro* [50–52]. These polyphenols include tannic acid (TA), myricetin (Myr), morin (Mor), quercetin (Qur), kaempferol (Kmp), (+)-catechin (Cat), and (−)-epicatechin (epi-Cat). The rank order of polyphenol activity, from most to least active, was TA >> Myr = Mor = Qur > Kmp > Cat = epi-Cat [50, 51]. Finally, a recent study showed that a commercially available polyphenol-containing grape seed extract, MegaNatural-AZ, significantly attenuated AD-type cognitive deterioration and reduced cerebral Aβ concentrations in Tg2576 mice [52]. The effects of this preparation appear to come from its antioxidant activity and from its ability to block Aβ folding and assembly [53].

b. Curcumin and Rosmarinic Acid—Curcumin (Cur), a potent polyphenolic antioxidant and anti-inflammatory agent [54–56], is a main component of the Indian curry spice turmeric. Rosmarinic acid (RA), another polyphenol, is an ester of caffeic acid and 3,4-dihydroxyphenyllacetic acid [57]. RA comes from the mint family of plants and possesses several interesting biological activities, including antioxidative, anti-inflammatory, anti-mutagenic, anti-bacterial, and anti-viral [58].

Employing Thioflavin T (ThT) assays and electron microscopy (EM), Ono *et al.* demonstrated that Cur and RA inhibited Aβ fibril formation and elongation and destabilized preformed fibrils [59]. Interestingly, both Cur and RA showed similar anti-amyloidogenic activity. Cur and RA possess two 4-hydroxy-3-methoxyphenyl rings and two 3,4-dihydroxyphenyl rings, respectively, separated by a short symmetric hydrocarbon chain that confers symmetry and compactness [60]. This structural arrangement may be critical for specific binding to free Aβ and for subsequent inhibition of Aβ fibril formation [59, 60]. Alternatively, this structure might mediate binding and destabilization of the cross-β core of Aβ fibrils [59].

Several reports suggest that Cur could be a useful AD therapeutic. In addition to data showing that Cur can protect cells from Aβ-induced oxidative injury [61], studies in Tg2576 mice show that Cur decreases the amounts of soluble and insoluble Aβ as well as lessening plaque burden in many affected brain regions [62]. Recently, Yang *et al.* also reported that Cur inhibits formation of Aβ oligomers and fibrils, binds plaques, and reduces plaque burden *in vivo* [63]. Moreover, systemic administration of Cur for 7 days to APPswe/PS1ΔE9 mice reduced existing plaques and caused significant reversal of structural changes in dystrophic dendrites [64]. In humans, phase II clinical trials of curcumin C3 complex (Cur, demethoxycurcumin, and bisdemethoxycurcumin) in persons with mild-to-moderate AD are ongoing [65].

c. Ginkgo Biloba—Ginkgo biloba leaves have been used for centuries in traditional Chinese medical practice [66]. Ginkgo leaf extract, which on average contain ~24% flavonol glycosides, ~6% terpene trilactones (ginkgolides, bilobalide), ~7% proanthocyanidins, and

other constituents, is prescribed for conditions that include cerebrovascular insufficiency, peripheral vascular insufficiency, and cognitive impairment associated with aging and AD [66]. Pre-clinical and clinical studies indicate that Ginkgo biloba extract counteracts neural and vascular damage, enhances cognition, improves blood flow, enhances tissue metabolism, and opposes the detrimental effects of ischemia [67, 68].

Ginkgo biloba extract has been reported to inhibit Aβ aggregation [69] and prevent Aβ-induced neurotoxicity *in vitro* [70, 71]. These effects may underlie results showing that chronic Ginkgo biloba treatment blocks age-dependent decline in spatial cognition in Tg2576 mice without altering Aβ levels or suppressing protein oxidation [72]. We note, however, that one study has shown that Ginkgo biloba treatment may inhibit Aβ production by lowering free cholesterol levels in aging rats [66].

Several clinical studies have suggested that Ginkgo biloba treatment may benefit patients with acquired cognitive impairment, including AD and vascular dementia, based on significant improvements in measures such as clinical global improvement (CGI), activities of daily living (ADL), and Neuropsychiatric Inventory (NPI) [73–75]. However, a recent DBRPCT involving 513 patients administered Ginkgo biloba did not show any treatment benefit [76]. It is possible that the lack of cognitive and functional decline in placebotreated patients in this study obscured any treatment difference resulting from the administration of Gingko biloba. Ongoing studies involving a broader spectrum of AD patients, from mild to severe, may help to determine whether Gingko biloba has therapeutic value [77].

d. Anti-Oxidants—Many studies indicate that oxidative injury is apparent in the brains of AD patients and that it may be involved in AD pathogenesis [78–80]. For this reason, a variety of antioxidants, including vitamin E (α-tocopherol) [81–85], vitamin C (ascorbic acid) [86, 87], and vitamin A [88, 89], have been suggested as potential therapeutic agents. In fact, administration of vitamins reportedly delays the progression of dementia [88, 90–94]. However, a recent study of vitamin E treatment of patients with mild cognitive impairment showed no benefits [95].

Several mechanisms have been postulated to explain the observed effects of vitamins, including protection of neurons from Aβ-induced neurotoxicity [81–87]. However, in addition to effects on redox chemistry, anti-oxidants also may affect Aβ assembly directly. To explore this possibility, Ono *et al.* [96] studied the effects of antioxidant vitamins, including vitamin A (all-*trans*-retinol, all-*trans*-retinal, and all-*trans*-retinoic acid), β-carotene, and vitamins B2, B6, C, and E on the formation, elongation, and destabilization of Aβ fibrils *in vitro*. Among these antioxidants, vitamin A and β-carotene inhibited the formation and elongation of Aβ fibrils and destabilized preformed fibrils. The rank order of inhibitory activity of the molecules examined was all-*trans*-retinol = all-*trans*-retinal > β-carotene > all-*trans*-retinoic acid. Consistent with the high potency of all-*trans*-retinol, Aβ40 fibrils pre-treated with all-*trans*-retinol have been found to be less toxic than untreated Aβ40 fibrils in MTT assays on HEK cells [96].

3. Peptidic Inhibitors

a. β-Sheet Breakers—Several reports have indicated that β-sheet-containing Aβ assemblies, including oligomers and fibrils, are neurotoxic [97–101]. It has been hypothesized that short peptides homologous to the central region of Aβ and with a low propensity to adopt a β-sheet conformation, "β-sheet breakers," could inhibit Aβ oligomerization and fibril formation. In particular, Soto *et al.* [102] showed that an 11-residue β-sheet breaker peptide (iAβ11) binds to Aβ with high affinity and inhibits Aβ fibril formation *in vitro*. A 5-residue β-sheet breaker peptide (iAβ5), with the primary structure LPFFD, later was synthesized and found to inhibit Aβ fibrillation, disassemble preformed fibrils, and prevent fibril-induced neuronal death in cell culture [103, 104]. Treatment with iAβ5, which has good pharmacological properties and rapid BBB penetration, reduced significantly the amyloid burden, neurodegeneration, and brain inflammation normally observed in two different Tg AD mouse models [105]. Chacón *et al.* [106] have reported that treatment with iAβ5 significantly improved hippocampal-dependent spatial learning (Morris water maze and working memory analysis) in rats injected with Aβ fibrils. Finally, Austen *et al.* [107] created two peptide-based inhibitors comprising the AβKLVFF sequence and flanking G and R residues (to increase solubility). These peptides, OR1 (RGKLVFFGR) and OR2 (RGKLVFFGR-NH_2), effectively inhibited Aβ fibril formation [107]. However, only the OR2 peptide also inhibited both Aβ oligomer formation and Aβ toxicity in human neuroblastoma SH-SY5Y cells [107].

b. N-Methyl Peptides—A fundamental aspect of Aβ fibril assembly is the formation of extended β-sheets. This suggests that disruption of interatomic interactions mediating β-sheet formation could have therapeutic benefits. One approach to β-sheet disruption has been the creation of N-methyl-peptide inhibitors. Methylation of backbone amides eliminates hydrogen atoms necessary for hydrogen bonding and prevents the close approach of other β-strands in a β-sheet through steric hindrance [108–112].

Incorporation of one N-methyl peptide as the edge strand of a β-sheet blocks subsequent addition of new strands [113]. Sciarretta *et al.* [114] have reported that incorporation of N-methyl amino acids into Aβ can disrupt both N- and C-terminal β-sheets and that this disruption renders Aβ "monomeric" and unable to form fibrils. In another study, using ThT fluorescence, EM, and MTT assays, Yan *et al.* [115] showed that an islet amyloid polypeptide (IAPP) analogue, [N-methyl-G24, N-methyl-I26]-IAPP (IAPP-GI), blocks formation of cytotoxic Aβ40 oligomeric and fibrillar assemblies. Decreases in the concentrations of these assemblies correlate with the formation of high-affinity Aβ40-IAPP-GI heteroassemblies [115].

4. Carbohydrate-Containing Compounds

a. GM1 Ganglioside—Ganglioside GM1, a glycosphingolipid localized to the outer leaf of the plasma membrane, exists at highest concentration in the brain. It has been suggested that GM1 and other gangliosides have potential as therapeutic agents in stroke and AD [116]. For example, GM1 administered intravenously to Tg PS/APP double transgenic mice reduces amyloid plaque load [117]. It is surprising then that GM1 ganglioside-containing vesicles have been found to associate avidly with Aβ and act as a template or "seed" that

accelerates the rate of Aβ fibrillation *in vitro* [118–121]. The observed reductions in cerebral plaque load caused by GM1 thus may result from GM1 acting as a "peripheral sink" for Aβ, as discussed in section 1 regarding antibodies. This hypothesis is reasonable considering that GM1 likely cannot cross the BBB [122].

Clinical trials have assessed the utility of GM1 as an AD therapeutic. A DBRPCT trial of 12 AD patients injected daily (i.m.) with GM1 failed to show significant improvement in cognitive performance [123]. However, in another study, but one that was "open label" and lacked a placebo control, patients did exhibit improved motor performance, delayed deterioration of cognitive tasks, increased cerebral blood flow, and improved neuropsychological evaluation. In this study, five AD patients receiving intracerebroventricular injections of GM1 (20–30 mg/24h) were treated for one year [124]. Based solely on the results of these two small studies, it appears that peripheral administration of GM1 may be ineffective. However, the value of intracerebroventricular GM1 injection also is uncertain, pending follow-up studies that integrate rigorous controls and appropriate cognitive assessments of AD with a larger patient pool.

b. Glycosaminoglycans (GAGs)—Proteoglycans (PGs), glycosaminoglycan (GAG) chains attached to a protein core, are complexes found within plaques in AD patients [125]. PGs bind avidly to Aβ, accelerate fibril formation, stabilize fibrils, and protect Aβ against proteolysis [126–128]. It is believed that low molecular weight GAGs have the ability to antagonize the action of PGs [127, 129]. For example, sulphonate or sulphate-containing GAGs inhibit the aggregation and toxicity of Aβ, presumably through a mechanism involving competition for Aβ binding sites with structurally analogous GAG side chains on PGs [130–132]. These activities suggest that small GAGs may be useful therapeutic agents. However, no clinical studies of this question have been reported yet.

5. Polyamines

Polyamines are low molecular weight aliphatic amines. The naturally-occurring polyamine spermine (H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2) has been reported to scavenge free radicals generated by Aβ that cause oxidative stress. However, treatment of cultured neurons with spermine and freshly prepared Aβ42 resulted in more toxicity than either spermine or Aβ alone [133]. Another polyamine with multiple medical applications, quinacrine (also known as Atabrine™), is a more promising potential inhibitor of Aβ assembly. Quinacrine has been investigated extensively for the treatment of prion diseases and has been shown to inhibit prion replication *in vitro* [134]. Recently, a multimeric quinacrine conjugate showed direct inhibition of Aβ self-assembly [135]. However, monomeric quinacrine showed no inhibitory effect on Aβ fibril formation [135]. Multimeric quinacrine compounds, and other polyamines, thus may comprise a new class of Aβ assembly inhibitors.

6. "Drug-Like" Compounds

"Drug-like" compounds are small molecules with physical and biological properties similar to those of drugs currently in use, which makes them attractive candidates for full-scale development efforts. These compounds contain functional groups or have physical properties that produce good absorption, distribution, metabolism, excretion, and toxicity profiles. A

number of metrics are used to evaluate attractiveness of candidate drugs. One is Lipinski's "rule of five" [136], which states that an orally active drug should not violate more than one of the following criteria: (1) no more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms); (2) no more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms); (3) a molecular weight < 500; and (4) a partition coefficient (log *P*) < 5. Other metrics include the number of rotatable bonds (≤8) and rings (≤4) [137]. Ghose *et al.* [138] have suggested that ideal ranges for the partition coefficient and molecular weight are −0.4–5.6 and 160–480, respectively. Ghose *et al.* also suggest a molar refractivity range of 40–130 m^3 mol^{-1}. "Drug-likeness" is a quality that is used frequently in the context of high-throughput screening (HTS) of combinatorial compound libraries [139].

a. "Drug-Like" Compounds Targeting A—A diversity of organic compounds inhibit Aβ fibrillogenesis *in vitro*, including nicotine [140], β-cyclodextrin [141], hemin and related porphyrins [142], the anthracycline 4′-iodo-4′-deoxydoxorubicin [143], hexadecyl-N-methylpiperidinium bromide [144], rifampicin [145], (−)-5,8-dihydroxy-3R-methyl-2R-(dipropylamino)-1,2,3,4-tetrahydronaphthalene [146], and melatonin [147]. Salvianolic acid B inhibits Aβ fibril formation, disaggregates preformed fibrils, and protects against Aβ-induced cytotoxicity [148].

In one study, Bartolini *et al.* [149] induced Aβ aggregation using human recombinant acetylcholinesterase (AChE) and then small molecules were tested for their ability to inhibit this process. Molecules including propidium iodide (a peripheral anionic-site ligand of human recombinant AChE), decamethonium bromide, donepezil, and physostigmine were active [149]. Propidium iodide, decamethonium bromide, physostigmine, and donepezil all are AChE inhibitors and four of the five FDA-approved therapeutic agents for AD belong to this drug class. Unfortunately, a relatively long history of use of these compounds in AD patients shows that they are not effective disease-modifying agents.

Non-steroidal anti-inflammatory drugs (NSAIDs) also have been reported to inhibit human aluminum-induced Aβ and IAPP aggregation *in vitro* [150, 151]. Kim and Lee found that 1,2-(dimethoxymethano)fullerene specifically binds to the 16–20 region of Aβ with high affinity, thus putatively inhibiting the early stages of amyloid formation [152]. The lack of structural similarity amongst the aforementioned drug-like compounds is striking, suggesting that these inhibitors bind to different sites on the Aβ monomer or to different structures formed by Aβ oligomerization or polymerization. This phenomenon contrasts with the actions of most other drugs, which depend on binding of one or more members of a class to a single target site. The diversity of Aβ structures to which the "drug-like" compounds bind complicates the execution of rational drug design strategies.

b. Tramiprosate—Tramiprosate (3-amino-1-propanesulfonic acid; Alzhemed™) has been shown to bind preferentially to pre-fibrillar (monomeric and oligomeric) forms of Aβ, preventing their conversion into higher-order aggregates and fibrils [153, 154]. Studies in primary neuronal cultures from rat brain showed that tramiprosate decreased Aβ42-induced cell death by 38% [153]. Administration of tramiprosate to Tg mice that develop cerebral amyloidosis resulted in significant reductions in plasma Aβ (60%), soluble Aβ40 (30%), insoluble Aβ40 (31%), soluble Aβ42 (25%), and insoluble Aβ42 (22%) [153]. Importantly,

tramiprosate crosses the BBB and causes minimal toxicity in mice [153]. For these reasons, clinical trials of tramiprosate were undertaken. Unfortunately, the latest results from a phase III trial in patients with mild-to-moderate AD showed no significant benefits relative to placebo-treated controls [155]. Neurochem, the maker of Alzhemed™, has abandoned the study and apparently now is marketing this product as a "dietary supplement" [156].

c. Nicotine—An inverse correlation has been reported between the amount of cigarette smoking and the risk of AD [157]. Although tobacco is linked with cancer and many other health risks, an active ingredient in tobacco, nicotine, may be a beneficial pharmaceutical agent [158]. Nicotine, injected or introduced into the body using a skin patch, was shown to improve significantly learning and memory in animals [159, 160] and in patients with AD [161–163]. These nicotine-related positive effects were ascribed mainly to the neuroprotection conferred against Aβ-induced toxicity by upregulation of nicotinic receptors deficient in the AD brain [164, 165]. However, nicotine also has been found to inhibit Aβ fibril formation and destabilize preformed fibrils [140, 166]. In mice, as would be predicted from these earlier studies, Nordberg *et al.* [167] showed that chronic nicotine treatment caused a significant diminution in Aβ42-containing plaques in Tg2576 mice.

The anti-amyloidogenic properties of nicotine may originate from the β-sheet disrupting character of the N-methylpyrrolidine substituent. This notion is supported by the facts that N-methylpyrrolidine has similar anti-amyloidogenic properties to nicotine [166] and the N-methyl and 5′-methylene pyrrolidine moieties appear to bind to the His residues of Aβ(1–28) [140]. If this supposition is valid, then nicotine derivatives containing N-methylpyrrolidine moieties can be developed as agents that disrupt Aβ assembly, possibly avoiding the vasoconstrictive and thrombogenic properties of nicotine.

7. Chaperones

Cellular chaperone proteins prevent the misfolding of nascent polypeptides. Chaperones selectively recognize and bind to exposed hydrophobic surfaces of non-native proteins *via* non-covalent interactions, thereby inhibiting aggregation of those proteins *in vivo* [168] and *in vitro* [169]. In addition to the prevention of protein aggregation, chaperones facilitate correct protein folding [168]. Consistent with these activities, numerous studies have suggested that heat shock proteins (HSPs) are critical for the prevention of amyloid formation [170–175].

Molecular chaperones comprise several distinct classes of sequence-conserved proteins, most of which are stress-inducible HSPs. Major classes of these HSPs are Hsp100, Hsp90, Hsp70, Hsp60, and the small HSPs. Data from cell-free *in vitro* studies indicate that Hsp70 and Hsp90 inhibit early stages of Aβ42 aggregation [169] and that Hsp90 or Hsp70, with its co-chaperone Hsp40, dissociate preformed Aβ oligomers but not Aβ fibrils. The small heat shock proteins Hsp20, Hsp27, and αB-crystallin bind Aβ40 and inhibit Aβ aggregation and toxicity [176]. Other reports show that Hsp20 [177] and Hsp104 [178] also limit amyloid formation *in vitro* in neuronal cells and yeast, respectively. *In vivo*, chaperones have been shown to block formation of Aβ aggregates in Tg *C. elegans* [179].

Graef *et al.* [180, 181] have integrated the chaperone function of the FK506-binding protein (FKBP), a large soluble molecule that binds to the immunosuppressant FK506 and inhibits the enzyme calcineurin, with the amyloid binding activity of Congo red (CR) by linking the two through a synthetic ligand for FKBP (SLF). In this complex, CR putatively caps amyloid fibrils while FKBP sterically blocks the large contact surface of fibrils, thereby preventing their interaction and further assembly. SLF-CR/FKBP and SLF-benzidine-CR/FKBP complexes both blocked Aβ aggregation and inhibited the neurotoxic effects of such aggregates in primary cultures of rat hippocampal neurons [180].

It has been suggested that an imbalance between the capacity of chaperones to mediate proper protein folding and the number of nascent misfolded proteins is the cause of cytotoxicity [173]. Increased chaperone expression thus might suppress Aβ aggregate-induced neurotoxicity. Clinical studies of such treatments have not been done. Until they are, techniques can be developed that enhance chaperone activity or increase chaperone expression. It is possible that these approaches, in addition to ameliorating Aβ-specific pathologic aggregation, might also have beneficial effects on the folding of other proteins, including those involved in other neurodegenerative diseases.

8. Metal Chelation

Alterations in brain metal ion homeostasis, particularly of Cu^{2+} and Zn^{2+}, have been implicated in Aβ-associated pathology [182]. Cu^{2+} and Zn^{2+} levels are elevated markedly in amyloid plaques, where the abundant Aβ present can coordinate these metal ions [183, 184]. Iron also has been found in Aβ deposits, and *in vitro* experiments indicate that all three metals, when coordinated by Aβ, may participate in redox chemistry leading to the production hydrogen peroxide and pathologic sequelae [185]. A logical therapeutic approach that has evolved from these findings is the sequestration of metals by chelation agents.

Bush *et al.* [186] have shown that chelators inhibit hydrogen peroxide production by Aβ *in vitro*. These compounds also can reverse the aggregation of Aβ *in vitro* and *in vivo* (based on analysis of post-mortem human brain specimens) [186]. A particularly important chelator is clioquinol (CQ), an analogue of 8-hydroxy-quinoline. CQ has moderate affinity for Cu^{2+} and Zn^{2+} and has been found to inhibit metal-induced Aβ aggregation and reactive oxygen species generation *in vitro* [187]. Oral administration of CQ to Tg2576 mice reduced brain Aβ burden by ~50% after 9 weeks [187].

In a pilot phase IIa trial of CQ (PBT1), AD patients showed evidence of delayed cognitive deterioration and significantly lowered plasma Aβ levels [188]. However, phase II/III trials were halted due to impurities in the formulation [189]. Adlard *et al.* [190] characterized a second-generation 8-hydroxy-quinoline analogue, PBT2, which also targets metal-induced Aβ aggregation and has greater BBB permeability. Compared to CQ, PBT2 significantly decreased soluble interstitial brain Aβ levels in Tg mice within hours and improved cognitive performance to levels that were equal to or better than those of non-transgenic littermates. Non-transgenic mice were unaffected by PBT2 administration.

A recent 12-week phase IIa DBRPCT of PBT2 was conducted on patients with mild-to-moderate AD [191, 192]. In this trial, participants received oral PBT2 (50 mg or 250 mg) or

placebo. Patients treated with 250 mg PBT2 showed significant diminution in frontal lobe functional deficits, as measured by the Category Fluency Test and Trail Making Part B, and significant decreases in CSF Aβ42 levels. Importantly, no serious side-effects were noted in patients that received the PBT2 formulation. Future trials on a larger AD patient pool will be necessary to determine the clinical utility of PBT2.

9. Osmolytes

Osmolytes are small organic solutes produced by cells to prevent the denaturation of intracellular proteins by environmental stressors such as heat, dehydration, high salt or urea concentration [193, 194]. Osmolytes are chemically diverse and include chemical classes such as sugars, polyols, amino acids, and methylamine compounds [193, 195]. At high concentrations, which generally are toxic in humans, osmolytes facilitate correct protein folding and therefore function as “chemical chaperones.” However, compounds that bind specifically to target proteins may function like osmolytes and do so at low concentrations. These “pharmacological chaperones” could be attractive agents for prevention of pathologic Aβ folding and assembly [196, 197].

Some studies of osmolytic effects on Aβ aggregation have been done. Trehalose, a simple α-linked disaccharide, is an effective inhibitor of Aβ aggregation and reduces Aβ-induced cytotoxicity [198]. Sucrose delays Aβ fibril growth and also hinders the racemization of D-aspartic acid [199], which contributes to the production of Aβ deposits [200].

The best studied osmolyte is cyclohexanehexol (inositol), a naturally-occurring cyclic polyol compound that is found in foods such as nuts, beans, and fruits. McLaurin *et al.* [201, 202] showed that three cyclohexanehexol stereoisomers, scyllo-cyclohexanehexol, epi-cyclohexanehexol, and myocyclohexanehexol, inhibit Aβ fibril assembly, disassembled preformed fibrils, and blocked oligomer-induced toxicity in primary neuronal cultures. Although myo-cyclohexanehexol is the most abundant isomer found in the brain, scyllo-cyclohexanehexol and epi-cyclohexanehexol were shown to be better inhibitors of Aβ aggregation and toxicity [202]. Furthermore, orally administered scyllo-and epi-cyclohexanehexol stereoisomers block the deposition and neurotoxic effects of Aβ in the TgCRND8 mouse model of AD [203]. These isomers also inhibited the formation of soluble Aβ oligomers and insoluble Aβ aggregates in a dose-dependent manner and decreased AD-like behavioral deficits, AD-like pathology, and mortality in the TgCRND8 mice [203]. Scyllo-cyclohexanehexol inhibited the growth of plaques in mice with advanced stages of AD-like pathology and brain concentrations of the compound could be achieved that were an order of magnitude higher than that of the endogenous compound [204]. Inosose stereoisomers, which differ from inositols by the replacement of one hydroxyl group by a ketone, also can inhibit Aβ aggregation [205].

It has been hypothesized that the mechanism of inositol and inosose stereoisomer action is osmolyte modulation of Aβ folding [206] and competition for Aβ binding sites. Inositol and inosose stereoisomers can compete for Aβ binding sites [203] with phosphatidylinositol lipids that facilitate Aβ oligomerization and fibril formation [201, 207–209]. However, in addition to these direct effects on Aβ aggregation, osmolytes may increase expression of HSPs and other intracellular chaperones [210] and increase the efficiency of protein folding

during cellular stress [211, 212]. In conclusion, cyclohexanehexols are attractive therapeutic agents as they readily cross the BBB, are found endogenously in the brain, and can diminish Aβ accumulation and neurotoxic effects before, during, and even after the onset of AD-like pathological events in mice.

10. Nascent Approaches

Thus far, we have reviewed therapeutic approaches for controlling Aβ assembly for which substantial pre-clinical or clinical data exist. We now turn to three nascent, but intriguing, approaches that also may have promise.

a. γ-secretase Modulators (GSMs)—As discussed in the introduction, a major therapeutic strategy for AD is blocking Aβ production. To do so, inhibitors are being sought that target the enzymes that produce Aβ, particularly β-secretase and γ-secretase, which cleave the N-terminus and C-terminus of Aβ, respectively, from APP. Until recently, small-molecule γ-secretase modulators (GSMs), such as tarenflurbil (also known as R-flurbiprofen or Flurizan™), have been hypothesized to target γ-secretase, although no clear mechanism of action has been delineated [213–215]. However, it recently has been demonstrated that enzyme inhibitors appear to bind APP directly [216]. Furthermore, the GSM interaction with APP was isolated to the 28–36 region of Aβ. Experimental evidence suggests that the N-terminal portion of this region is a critical mediator of Aβ folding and aggregation [217] and that this region would be a particularly important therapeutic target [218]. An unanticipated application for GSMs thus may be as inhibitors of Aβ folding and aggregation. The recognition of this novel use of GSMs is particularly fortuitous in light of recently presented results of phase III clinical trials of Flurizan™ that failed to show any benefit in AD patients [219].

b. Aptamers—Aptamers are short single-stranded DNA or RNA molecules that bind to various substrates, including nucleic acids and proteins, with high specificity and avidity. For example, an RNA aptamer specific for murine PrP^{Sc} possessed a dissociation constant of ~2.1 nM [220] and many other protein-specific aptamers have affinities in the nM range [221–223] to pM range [224]. Aptamers are produced from screens of large combinatorial libraries in a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) that identifies and amplifies structures with high target affinity [225]. Aptamers may prove to be exceptionally useful agents for preventing or reversing the peptide aggregation and fibril formation associated with AD. Aptamers have some advantages over antibodies. Antibodies may be difficult to produce if the antigens being targeted are toxic or weakly immunogenic [224]. In addition, murine antibodies, which are the most common source of monoclonals, necessitate “humanization” for therapeutic use [224]. Aptamer synthesis, in contrast, does not have these difficulties. Aptamer synthesis is straightforward, cost-efficient, and amenable to automation [224]. In the AD field, RNA aptamers recently have been developed that specifically modulate protein factors that interfere with the recruitment of proteins that typically associate with BACE1 (β-secretase) [222].

c. Platinum-Based A Inhibitors—Aβ amino acid residues His6, His13, and His14 form coordination complexes with certain metals that accelerate Aβ assembly and are involved in

redox chemistry [226, 227]. As discussed above, metal-targeted strategies, e.g., the use of chelators, is one approach to blocking the coordination process and its sequelae. However, rather than target the metal itself, an alternative approach recently discussed by Barnham *et al.* [228] is the use of various 1,10-phenanthroline-$PtCl_2$ complexes to bind to the high affinity metal coordination sites on Aβ. In an impressive set of experiments, Barnham *et al.* demonstrated that this binding alters Aβ structure, inhibiting fibril formation, production of reactive oxygen species, perturbation of cellular redox activity, and synaptotoxicity.

CAVEATS AND CONCLUSIONS

An unprecedented number of clinical trials are being conducted to identify AD drugs. The diversity of potential therapeutic agents (Table 1) reflects the complexity of the disease mechanism(s) (Fig. 1). Initial trails have been disappointing and thus the long awaited first disease-modifying therapeutic agent remains elusive. Such disappointments are not unusual, but they should stimulate discussion about current therapeutic strategies. Two topics are especially important: (1) is the amyloid cascade hypothesis correct; and (2) if it is, are the therapeutic targets extant the correct ones?

The answer to the first question is unknown. However, the innovative and complementary therapeutic strategies currently being executed may provide one. If efficacious drugs are discovered, support for the amyloid cascade hypothesis will have been strengthened and impetus for increased efforts to develop Aβ-specific therapeutics will exist. More importantly, patients finally would have ameliorative or curative therapeutic options.

If efficacious drugs are not discovered, both questions will remain. In that case, continued efforts to develop Aβ-specific drugs also are warranted to ensure that all reasonable targets are studied. These efforts must be conducted thoughtfully and rigorously, and if they are, the resultant information either will lead the field in more promising directions or provide compelling reasons for a shift to new working hypotheses of disease causation.

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ABBREVIATIONS

Aβ	Amyloid β-protein
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADAS-Cog	AD Assessment Scale-Cognitive Subscale
APP	Amyloid β-protein precursor

BBB	Blood-brain barrier
Cat	(+)-Catechin
CQ	Clioquinol (iodochlorhydroxyquin or 5-chloro-7-iodo-8-hydroxyquinoline)
CR	Congo red
Cur	Curcumin
DAD	Disability Assessment Scale for Dementia
DBRPCT	Double-blind, randomized, placebo-controlled trial
EM	Electron microscopy
Epi-Cat	(−)-Epicatechin
FKBP	FK506-Binding protein
GSMs	γ-Secretase modulators
HEK	Human embryonic kidney
HSP/Hsp	Heat shock protein
iAβ	β-Sheet breaker peptide
ICAD	International Conference on Alzheimer's Disease
i.m.	Intramuscular
i.v.	Intravenous
Kmp	Kaempferol
MHC	Major histocompatibility complex
Mor	Morin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myr	Myricetin
OR1	Peptide with the sequence RGKLVFFGR
OR2	Peptide with the sequence RGKLVFFGR-NH_2
PC12	Pheochromocytoma 12
PrP^{Sc}	Scrapie form of the prion protein
Qur	Quercetin
RA	Rosmarinic acid
SLF	Synthetic ligand for FKBP

TA	Tannic acid
Tg	Transgenic
Tg2576	Transgenic mouse that over-expresses human APP with the Swedish (K670N, M671L) mutation
TgCRND8	Transgenic mouse that over-expresses human APP with Swedish (K670N, M671L) and Indiana (V717F) mutations
ThT	Thioflavin T

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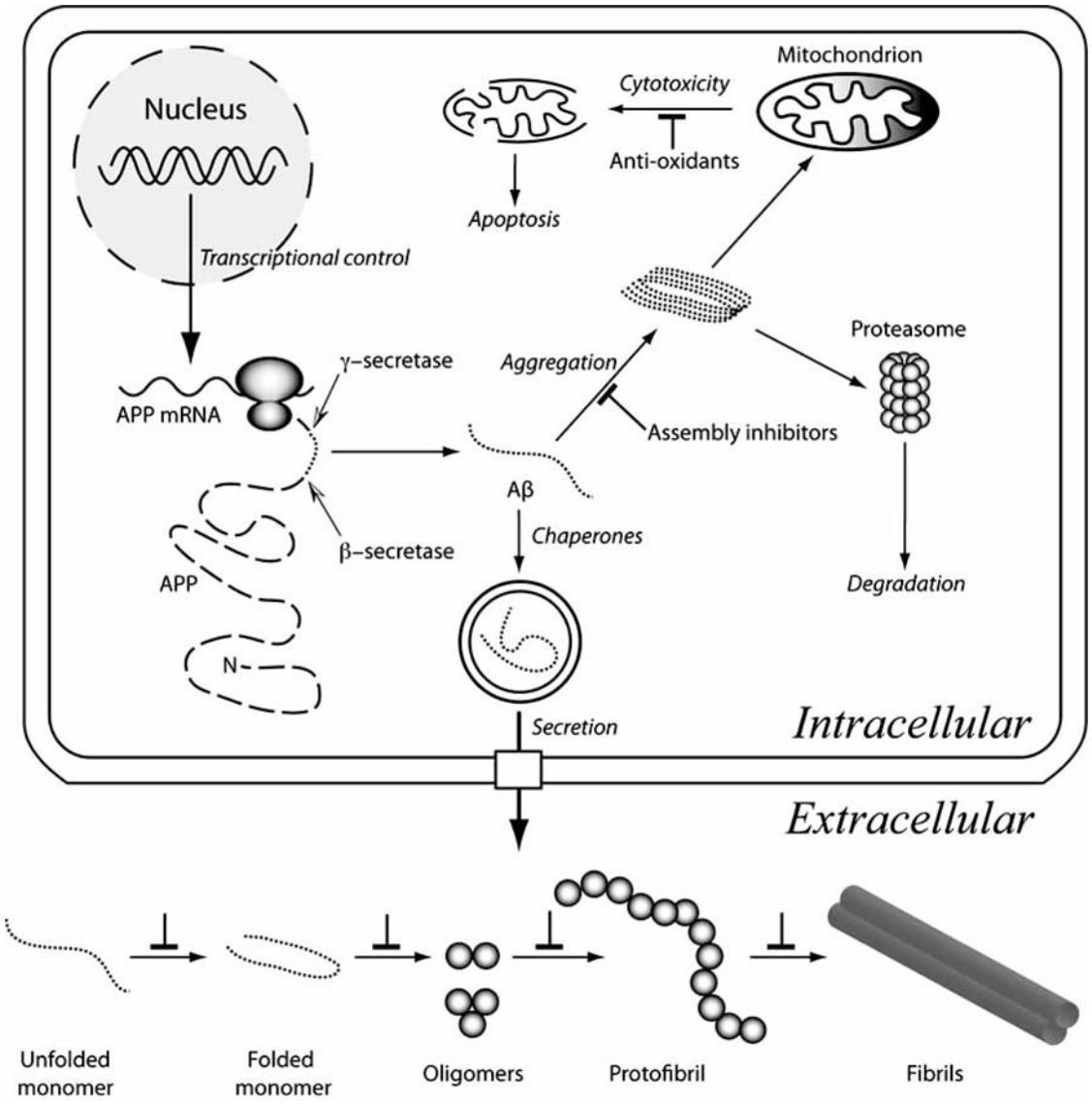

Fig. (1).

Aβ metabolism and assembly. Aβ (dotted lines) is produced by the sequential endoproteolytic cleavage of APP (dashed line). β-secretase cleavage (black-white arrowhead) produces the Aβ N-terminus, after which γ-secretase (black-white arrowhead) releases the Aβ C-terminus from APP. Transcriptional, translational, and endoproteolytic events all are targets for therapies to block Aβ production. The unstructured Aβ monomer may fold or aggregate intracellularly to produce toxic assemblies. One postulated cytotoxic mechanism is mitochondrial injury, which produces reactive oxygen species, mitochondrial injury, and apoptosis. Anti-oxidants could ameliorate redox effects directly. Assembly inhibitors would block this and other effects arising from formation of pathologic assemblies. Folding chaperones also would assist in this process. Aggregates may be eliminated through proteasomal digestion, but saturation of this system would result in cytotoxicity. Aβ secretion is a normal cellular process. Extracellular assembly of Aβ may occur in a variety of milieus. The micromolecular (pH, chemical composition) and

macromolecular (proteins, lipids, carbohydrates) characteristics of these milieus differ, thus Aβ assembly pathways and kinetics also are likely to differ. Nevertheless, *in vitro* and *in vivo* experiments suggest that Aβ proceeds along a linear pathway comprising many populated monomer conformational states, a population of partially folded states (some of which facilitate peptide oligomerization), a more restricted distribution of oligomers (with distinct distributions for Aβ40 and Aβ42), protofibrils, and fibrils (of which multiple morphologies exist). Each of the inter-state transitions is a potential therapeutic target (⊥ symbol).

Table 1

Current Aβ Assembly Therapeutics

Section	Therapy	Mechanism	Example(s)	Clinical Trials
1a	Active immunization	Aβ assembly, phagocytosis (antibody-dependent and independent), peripheral sequestration/elimination of cerebral Aβ, neutralizing antibodies	AN1792 ACC-001 CAD-106 V-950	Phase II[a] Phase II[b] Phase I[c] Phase I[d]
1b	Passive immunization		Bapineuzumab LY2062430 IVIg RN-1219 R-1450	Phase III[e] Phase II[f] Phase II[g] Phase I[h] Phase I[i]
2	Nutraceuticals	Aβ assembly, anti-oxidant	Curcumin Ginkgo biloba	Phase II[j] Phase III[k]
3	Peptidic inhibitors	Aβ assembly	β-sheet breakers N-methyl peptides	None
4	Carbohydrate-containing compounds	Aβ assembly	GM1 ganglioside	None
5	Polyamines	Aβ assembly	Quinacrine	None
6	“Drug-like” compounds	Aβ assembly	Alzhemed™ Nicotine	Phase III[l] None
7	Chaperones	Aβ assembly	Hsp20, Hsp27, Hsp70, Hsp90	None
8	Chelation	Aβ assembly, metal sequestration	Clioquinol PBT2	Phase II/III[m] Phase II[n]
9	Osmolytes	Aβ assembly	Cyclohexanehexol	None

Trials

[a] failed;

[b] stopped [20, 21];

[c] ongoing [22];

[d] ongoing [23];

[e] ongoing [24–27];

[f] ongoing [28];

[g] ongoing [35];

[h] ongoing;

[i] ongoing [36];

[j] ongoing [65];

[k] ongoing [77];

[l] failed [155];

[m] temporarily halted due to impurities in the formulation [189];

[n] ongoing [191, 192].