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Amyloid beta-peptide aggregation. What does it result in and how can it be prevented?

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1. ABSTRACT

Polymerisation of the amyloid beta-peptide (Abeta) gives rise to oligomers and amyloid fibrils, processes that generate cytotoxic assemblies and are associated with neuronal dystrophy and development of Alzheimer's disease. The relationship between Abeta aggregation and the development of Alzheimer's disease has resulted in immense efforts to find ways to prevent it. In spite of this, therapeutic approaches with proven clinical efficacy remain to be identified. The lack of success so far probably stem from a combination of factors. The details of the Abeta aggregation process (es) are not known, in particular several oligomeric forms have been identified but are not yet defined at a molecular level, Abeta is structurally polymorphic which complicate identification of compounds that bind selectively and strongly, and it is not settled which Abeta species is the main disease causing agent. Herein we review current knowledge about monomeric, oligomeric and polymeric Abeta, and discuss ongoing attempts to identify aggregation inhibitors and problems associated therewith.

2. INTRODUCTION

Amyloid formation is a key step in the development of several neurodegenerative diseases. Alzheimer's disease is the most common form of dementia and the single greatest risk factor for development of Alzheimer's disease is old age (1). World wide the elderly population is increasing and with it the number of patients suffering from this severe and devastating disorder. The efforts to try to in detail understand the mechanisms that cause the disease have been immense, both from academic laboratories as well as from pharmaceutical companies. This has rendered a greater understanding of the disease and more potential therapeutic agents than ever are now in clinical trials. However, so far no causative treatment is available, only treatments that affect the symptoms. The details of what causes the disease have been a matter of debate and several hypotheses have been suggested. This article tries to give an overview of where the research field is today with respect to amyloid β-peptide (Aβ) formation, aggregation and the neurotoxicity it exerts, as well as the current efforts to find ways to inhibit this process.

Alzheimer's disease comprises two distinct neuropathological hallmarks in the brain: aggregated Aβ in amyloid plaques and hyperphosphorylated tau in neurofibrillary tangles (2). The amyloid plaques are extracellular lesions surrounded by activated microglia and reactive astrocytes and are thought to precede the formation of hyperphosphorylated tau which occurs within neurons. Aβ, which is the main component of the amyloid plaques, is a partly hydrophobic peptide that is produced by sequential cleavages of the amyloid precursor protein (APP) by β- and γ-secretase (Figure. 1). The Aβ peptide corresponds to a major part of the transmembrane domain of APP, which likely accounts for its aggregation prone behaviour. Aβ is overall amphiphilic since residues 1-16 are hydrophilic while residues 17 to 40/42 are mainly hydrophobic. The γ-secretase protease shows low substrate sequence specificity and generates Aβ peptides of varying length, with the main product being 40 residues long. C-terminally elongated Aβ consisting of 42 amino acids is generated to a lower extent, but this form is more prone to aggregate. It is this longer variant of Aβ that is the most prominent peptide species within the amyloid plaques (3) .

The understanding of the events underlying the formation of Aβ fibrils has greatly increased, and the toxicity that Aβ and assemblies thereof exert has been in focus for the research field for the last decade. The endpoint stage of Aβ oligomerisation and aggregation, the amyloid fibrils, were earlier thought to be the major culprit to the disease since it is this form of Aβ that is found in the Alzheimer plaques. It is now generally recognised, however, that the lower molecular weight forms of Aβ aggregates are more toxic to cells and synapses. This theory is described in the amyloid cascade hypothesis which is outlined in Figure 2 (4). The hypothesis suggests that Aβ aggregation and accumulation cause cell toxicity and eventually leads to development of Alzheimer's disease. The amyloid cascade hypothesis was first presented in 1992 and has since then been modified to emphasize the importance of early species in the aggregation pathway (5). Recently, Meyer-Luehmann *et al* (6) showed in mutant APP transgenic mice (see below) that mature plaques surrounded by astroglia, microglia and dystrophic neurites can form within a few days from microplaques. This reinforces the view that Aβ aggregation and plaque formation drives disease progression and indicates that in the early stages of disease, microplaques can damage surrounding neurons within days. This is important information that has to be taken in considerations when developing *in vitro* and *in vivo* models and assays of Aβ toxicity. The genetic association most well characterised for Alzheimer's disease is allelic isoforms of apolipoprotein E. The ε4 allele is a major risk factor for the disease whereas the ε2 allele is protective (7). The importance of apolipoprotein E appears to be related to fibril formation of Aβ, where the isoforms show different potencies with regard to amount of fibrillar deposits generated (8).

As will be discussed in greater detail below, the Aβ aggregation process and its intermediates are difficult to define experimentally. This has led to the use of a number of *in vitro* protocols for their study (9, 10). Some of the *in vitro* methods employed are common in protein biochemistry in general, *e.g.* size exclusion chromatography, although the unusual properties of Aβ may pose special challenges, while other approaches have been designed specifically for the study of Aβ. Likewise, there are several animal models available for Alzheimer's disease. Although none of them correlate well with the disease observed in humans, they are useful in order to study certain aspects of the disease. By far the most widely used models are mice with transgenic expression of human APP containing one or several mutations causing familial Alzheimer's disease. Three examples are mice transgenic for the Swedish APP mutation (K670N/M671L, Tg2576 mice), transgenic mice expressing human APP containing the V717F mutation (PDAPP mice), and mice transgenic for both the Swedish and London (V717I) mutations (TgCRND-8 mice), see (11) for a review. In some models mutated human presenilin 1 (a γ-secretase catalytic component) is expressed together with APP, resulting in a more rapid manifestation of disease phenotype. APP transgenic mice display cognitive impairment that is evident after 4 months in the mice with strongest phenotype, or after up to 10 and 12 months of age for mice with a weaker phenotype (12). Amyloid plaque deposition is apparent in these mice and it occurs after the cognitive deficits can be measured. Drawbacks of most mouse models are that neuronal loss is marginal and that the tangles of hyperphosphorylated tau are not evident. However, in a triple transgenic mouse expressing human isoforms of mutated APP, presenilin and tau, both of the neuropathological hallmarks of Alzheimer's disease are present and cell loss is observed (13).

Another mammalian disease model available, but much more rarely used than the mouse models, is transgenic rats (14, 15). In addition, some studies have been performed where Aβ is injected intracranially in mice or rats. Non-mammalian models of the disease have been generated using zebra fish, *Danio rerio,* (16) and even invertebrates, i.e. *Drosophila melanogaster* and *Caenorhabditis elegans* (17). These disease models can of course not recapitulate all the aspects of a complex human neurodegenerative disorder. However, in terms of addressing specific aspects of the disease these models can be useful. *Drosophila* models, for example, have been used for studying certain aspects of Aβ mutations and their effects on aggregation properties (18, 19). In addition, short generation times and relatively short life spans of invertebrates enable large genetic and pharmacological screens to be performed. For example, the critically required γ-secretase components Aph-1 and Pen-2 were found in genetic screens of *C. elegans* (20, 21).

3. Aβ GENERATION AND POSSIBLE THERAPEUTIC TARGETS

Ever since the visualisation of amyloid plaques by Alois Alzheimer in 1906 (22) the Aβ aggregation process, as such, has been one of the most evident targets. However, this approach is difficult since tampering with

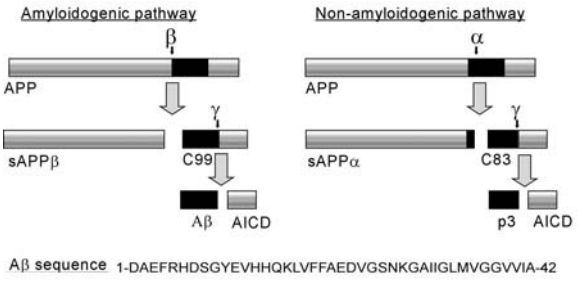

Figure 1. Illustration of the amyloidogenic (left panel) and non-amyloidogenic (right panel) processing pathways of APP. In the amyloidogenic pathway the aggregation-prone Aβ-peptide (black box) is produced by sequential cleavage by β- and γ-secretase. In the non-amyloidogenic pathway the p3 fragment is formed *via* processing by α- and γ-secretase. The extracellular ectodomain is liberated by the N- terminal proteases (β- and α-secretase), generating soluble APP-β and soluble APP-α (sAPPβ and sAPPα, respectively). AICD (APP intracellular domain) is the most C-terminal part of the protein released by γ-secretase cleavage. The amino acid sequence of human $A\beta_{1-42}$ is given at the bottom.

the aggregation propensity of the peptide might result in an increase of smaller oligomeric species, *e.g.* upon disruption of the amyloid plaques. If the Aβ peptide is not cleared properly, the oligomers might rather induce and accelerate the toxicity exerted on nearby neurons and synapses. Recently a phase III trial of an anti-Aβ aggregation compound, tramiprosate or aminopropane sulfonic acid, was abrogated due to lack of effect, even though a clear effect could be found *in vitro* and in TgCRND-8 transgenic mice (23). The reasons for the discrepancy between *in vitro* and animal experiments on the one hand and clinical studies on humans on the other are not known. However, there are several obvious differences; *in vitro* and in transgenic mice the Aβ levels are often much higher than in patients and short term effects are monitored, while in the patients, the amyloid plaques probably have accumulated and aged under decades. Irrespective of underlying reason, this illustrates what a difficult target the Aβ aggregation as such is. Reducing the amounts of Aβ available for aggregation, by preventing its formation from APP, could circumvent the problems associated with finding aggregation inhibitors. In APP transgenic mice, reducing the Aβ production might halt progression of pathology although existing plaques persist, suggesting that early treatment may be imperative (24).

APP is proteolytically processed by β-secretase and γ-secretase generating the N- and C-terminus of Aβ, respectively (Figure 1). β-Secretase can also cleave Aβ at position 11 (25). γ-Secretase is an enzyme complex with aspartyl protease activity critically requiring four different components (26). The presenilins (1 and 2) are transmembrane proteins which exert the catalytic activity *via* two aspartyl residues located in the membrane interior. The other members of the γ-secretase complex are Nicastrin, Aph-1 and Pen-2. The γ-secretase complex is ubiquitously expressed and has a wide range of substrates, *e.g.* the Notch receptor which is important in fetal development. Compounds that inhibit γ-secretase activity have been identified and as expected they reduce Aβ formation. γ-Secretase inhibitors are thus potential therapeutic agents in Alzheimer's disease, but they also interfere with Notch signalling resulting in adverse side effects such as gastrointestinal tract symptoms in mice (27). Modulation of the γ-secretase activity, as exerted by a subset of non-steroidal anti inflammatory drugs, therefore appears more promising than the approach of completely inhibiting the enzyme. The non-steroidal anti inflammatory drugs shift the specificity of the cleavage site in APP and thereby generate more of the less aggregation prone $A\beta_{1-38}$ while reduced amounts of $A\beta_{1-42}$ is produced without diminishing Notch signalling (28). Other aspects that can be considered when aiming for γ-secretase as a therapeutic target is to find compounds with a therapeutic window that allows a decrease in Aβ production enough to get a clinically relevant effect while for example the Notch signalling remains at a level where adverse side effects are avoided. γ-Secretase belongs to a family of related proteases that perform regulated intramembrane proteolysis (29). It is interesting to note that two other members of this family cleave Bri2, a transmembrane protein that can generate peptides, ABri or ADan, that form amyloid fibrils in familial British or Danish dementia (30).

The other major enzyme in prevention of Alzheimer's disease is β-secretase. This enzyme has narrower substrate specificity than γ-secretase which could make it a better target from a therapeutic point-of-view and knock-out in mice of the β-secretase enzyme, Bace, results in viable offspring (31). The crystal structure of Bace revealed that the enzyme contains a comparatively wide enzymatic cleft (32) and it has been suggested that the active site can be difficult to target because of its size. However, there are recent reports of compounds binding to the enzyme and reducing β-secretase activity (33, 34). In an alternative pathway (Figure 1) APP is instead processed by α- and γ-secretase and this generates the non-amyloidogenic fragment p3. The α-secretase cleavage site is within the Aβ sequence between residues 16 and 17, and p3 corresponds to Aβ residues 17-40/42. Finding ways of increasing the α-secretase cleavage could thus potentially reduce the amounts of Aβ produced. Recent progress in development of secretase inhibitors has been reviewed in (35) and this topic will not be further covered here.

4. STRUCTURES OF Aβ MONOMER AND POLYMERS

A rational approach to the design of Aβ aggregation inhibitors would optimally be based on structural knowledge at atomic resolution. Aβ is a structural chameleon that can take on different conformations depending on its environment (Figure. 3). For monomeric and soluble Aβ, as well as for Aβ in amyloid-like fibrils generated *in vitro*, structures have been determined at atomic resolution using NMR methods. In contrast, no

high-resolution experimental data have been published for any oligomeric species. This is frustrating as oligomeric Aβ assemblies are thought to constitute the main toxic species. The concept of structural chameleons was originally proposed for α-synuclein (36). α-Synuclein, like Aβ, lacks a well defined conformation; it can adopt different structures depending on environment and it can also form several different types of aggregates (36). Aggregation of α-synuclein is linked to neurodegenerative disorder, in particular Parkinson's disease. The overall similarities between Aβ and α-synuclein make it possible that the problems discussed herein as regards design of Aβ aggregation inhibitors also to some extent apply to other protein chameleons linked to human disease.

In water solution at slightly acidic pH Aβ lacks stable secondary structure, but deviates from a completely random coil by the presence of local, short-lived, non-random conformations (37). Structure calculations based on NMR-derived constraints yielded trends towards non-random structures for the peptide segments 8-12, 16-24, and 30-34. Residues 20-24 form a helical turn and the segment 16-24 shows several hydrophobic contacts between side-chains (Figure. 3). The segments 8-12 and 30-34 show turn-like structures. Interestingly, although $A\beta_{1-40}$ and $A\beta_{1-42}$ show different aggregation behaviour, where the latter is much more prone to aggregate, their solution structures are practically identical, except that $A\beta_{1-42}$ is less flexible in the region from residue 30 to the C-terminus (37).

In the presence of detergent micelles, *e.g*. SDS micelles, or structure-inducing organic solvents like trifluoroethanol or hexafluoroisopropanol, Aβ adopts more stable secondary structural elements (38). The structures determined in such environments differ slightly from each other, probably mostly depending on differences in exact solvent conditions and peptide fragments used, but collectively they show that the regions covering residues ~15-24 and ~29 to the C-terminus form α-helices. Jarvet *et al* (39) reported that in SDS micelles the helix covering residues 15-24 is superficially oriented and the face with residues 16, 20, 22, and 23 is oriented towards the surrounding solvent, while another helix covering residues 29-35 is buried in the hydrophobic interior of the micelle. Detergent micelles and organic co-solvents to some extent mimic the presence of a lipid membrane environment. It is therefore conceivable that the Aβ structures derived in the presence of such additives resemble the structure of the corresponding region in membrane-bound APP (Figure. 3).

In amyloid-like fibrils $A\beta_{1-40}$ (40) and $A\beta_{1-42}$ (41) adopt fundamentally different conformations than in water solution or in membrane-like environments. Major features are essentially the same for $A\beta_{1-40}$ and for $A\beta_{1-42}$ fibrils; they are both cross-β structures (i.e. the β-strands are perpendicular to the fibril axis and the hydrogen bonds between strands are parallel to the fibril axis (42)) composed of two-layered, parallel, and in-register β-sheets connected by a turn region. In $A\beta_{1-40}$ fibrils residues 12-24 make up one of the strands and residues 30-40 make up the other, while in $A\beta_{1-42}$ fibrils residues 18-26 and 31-42 form β-strands. In both cases, the two strands form contacts *via* side-chain interactions, but important differences are seen. In $A\beta_{1-40}$ fibrils the side-chain interactions are intramolecular, while in fibrils formed by $A\beta_{1-42}$ the upper sheet layer is displaced relative to the lower layer, so that the two β-strands of Aβ molecule *i* form intermolecular side-chain interactions with the strands of molecules *i*+1 and *i*-1, respectively (Figure. 3).

It is notable that the dominating secondary structure of soluble or membrane-bound Aβ is helical and that of fibrillar Aβ is β-strand, but the regions involved in secondary structures are largely the same for both states. The regions covering residues ~15-25 and ~30-40/42 form helices or strands, respectively, while residues ~1-14 are flexibly disordered irrespective of the nature of the remaining structure. It is interesting that $A\beta_{1-40}$ bound to a binding protein selected by phage disply adopts a β-hairpin structure where residues 17-23 and 32-37 form β-strands (43). From a structural point-of-view Aβ aggregation can be described as a folding transition that occurs when Aβ is released from APP, where it is in its transmembrane state, into an aqueous solution, whereby intramolecular helix interactions and helix-membrane interactions are eventually replaced with β-strand–β-strand interactions in amyloid-like fibrils. Whether the process resulting in this structural transition gives rise to cell toxic intermediates, or if such species instead are formed *via* alternative pathways and whether the fibrils as such contribute to cytotoxicity are still open questions.

5. Aβ ASSEMBLY AND TOXICITY

Detailed knowledge of how Aβ aggregates into fibrils and about the intermediates formed in the process is essential for a better understanding of Alzheimer's disease and how to prevent it. In the literature there is a large number of studies where different types of Aβ assemblies are reported. Early studies mainly focused on the formation and toxicity of mature Aβ fibrils since they were believed to be directly associated with neurotoxicity, but recently a number of studies have indicated that instead various prefibrillar, soluble oligomeric forms of Aβ are more likely to be the pathological species. This is supported by a weak correlation between the number of fibrillar deposits and severity of Alzheimer's disease (44, 45), and by more recent studies showing a better correlation between levels of soluble forms of Aβ and cognitive impairment (46, 47).

The term oligomeric Aβ is wide and generally includes all species that remain in aqueous solution after high-force centrifugation, and a large number of such forms has been identified, both *in vitro* and *in vivo*. Besides apparent differences between naturally and synthetically derived oligomers, there are also discrepancies depending on methods employed to generate and detect oligomers. So far it is not clear whether all oligomers identified are obligate intermediates on the Aβ fibrillation pathway, or if they are the products of competing processes (48). The two most abundant covalent forms of Aβ, $A\beta_{1-40}$ and $A\beta_{1-42}$, appear to undergo aggregation through slightly different pathways, thereby producing different early intermediates

Table 1. Survey of different forms of oligomeric Aβ assemblies described

Aβ assembly	System	References
Protofibrils	*In vitro*, synthetic Aβ	(50-52)
Annular structures	*In vitro*, synthetic Aβ	(54)
ADDLs (Aβ-derived diffusible ligands)	*In vitro*, possibly *in vivo*	(55, 59, 60)
Aβ*56	Tg2576 mouse model	(62)
Globulomers	*In vitro*, human brain, Tg2576 mouse model	(63)
Low-n oligomers (dimers, trimers, tetramers)	*In vitro*, human brain and CSF	(65-68)

49). Table 1 summarises oligomeric Aβ species that have been identified up till now. The oligomers are usually defined by their size and/or ultrastructural appearance but the nomenclature used is not uniform and some of the species may overlap or even be identical.

Protofibrils seem to be true intermediates on the pathway to fibrils and the largest form of soluble species. They are shorter (< 200 nm) and slightly thinner than mature fibrils (50, 51) and have been shown to induce neurotoxicity (52). In experiments with $A\beta_{1-42}$ the protofibrillar structure can be preceded by a beaded structure called paranuclei, which has not been observed for $A\beta_{1-40}$ (49). Nilsberth *et al* concluded that that one rare form of familial Alzheimer's disease is associated with increased protofibril formation due to a single point mutation in Aβ, resulting in replacement of Glu22 with Gly (53). Annular structures have been found when incubating synthetic Aβ. They are doughnut shaped, pore-like structures with an outer diameter of 8-12 nm and a molecular size around 150-200 kDa (54). ADDLs (Aβ-derived diffusible ligands) are smaller than both protofibrils and annular structures and were first found by Lambert *et al.* (55) when incubating synthetic Aβ1-42 in the presence of the protein clusterin, also known as apolipoprotein J. It is interesting to note that recently Wilson and co-workers have found that clusterin influences fibril formation by binding to prefibrillar species (56, 57). ADDLs are globular structures but their reported sizes differ depending on the analytical methods employed. They were first reported to be low-n oligomers (17-25 kDa, corresponding to 4-6 mers) using SDS-PAGE (55, 58) but a more recent study employing analytical ultracentrifugation indicate that they might be more heterogeneous with sizes up to around 200 kDa (59). ADDLs have been shown to inhibit hippocampal long-term potentiation (55) and Gong *et al.* have shown that the levels of ADDLs-like oligomers in human brain are markedly elevated in Alzheimer's disease brains compared to controls (60). Deshpande and colleagues recently provided an interesting comparison of Aβ high molecular weight oligomers, ADDLs and fibrillar Aβ (61). In this study they could show that Aβ oligomers exerted toxicity towards primary human cortical neurons within one day, for ADDLs the toxicity was evident after five days and fibrillar Aβ required higher concentration and it took 10 days before toxicity became evident. Both Aβ oligomers and ADDLs bound to synapses, indicating that this could be an important step in the toxicity pathway leading to neurodegeneration.

Lesné *et al.* (62) have reported an SDS-stable 12mer of Aβ, which they named Aβ*56 from its migration upon SDS-PAGE, in the brain of Tg2576 transgenic mice. The appearance of Aβ*56 correlated with the appearance of impaired long term memory in the mice. Similar memory impairment was seen when Aβ*56 was administered to healthy, young rats. Whether Aβ*56 is in fact an *in vivo* correlate to ADDLs remains to be seen. Barghorn *et al* (63) concluded that $A\beta_{1-42}$ can either polymerise *via* a classical nucleation-dependent pathway to fibrils, or, alternatively, form globular structures composed of 12 peptides. The latter, which were termed globulomers, were suggested to be build up in a micellar-like fashion with the hydrophobic C-terminal part of Aβ buried in the interior and the more hydrophilic N-terminal part facing the surrounding solvent. The globulomers could be formed *in vitro* and were also isolated from brains of Alzheimer's disease patients and Tg2576 transgenic mice. The globulomers inhibited long-term potentiation in rat hippocampal slices. Whether these globulomers are similar to the Aβ*56 species remain to be established.

Recently, more attention has been focused on low-n oligomers, dimers up to tetramers (64). These are SDS-resistant oligomers and are apparently secreted by cultured cells (65, 66). They can be isolated from an APP transfected cell line and from human brain and cerebrospinal fluid (67). These oligomers have been shown to inhibit hippocampal long term potentiation when injected into the brain of rats (68) and their effect on memory and synapses, as well as possible correlation with disease initiation and progress, are currently being investigated.

6. INHIBITORS OF Aβ AGGREGATION AND TOXICITY

Since the discovery that the amyloid in Alzheimer's disease mainly is composed of Aβ (69) researchers have tried to gain better insight into the mechanism of Aβ aggregation and find ways to inhibit it. As outlined above, a number of Aβ assemblies have been proposed to exert neurotoxic effects, but lack of evidence for which forms that are the pathological species *in vivo* and the scarcity of structural data on the oligomers complicate a rational search for compounds that could inhibit Aβ aggregation and toxicity. In spite of theses obstacles, a number of different compounds that interfere with Aβ aggregation in one way or another have been published. The currently pursued strategies to reduce Aβ aggregation and toxicity, apart from secretase inhibition (see above), can be broken down to immunotherapy and inhibition or alteration of aggregation by low molecular weight compounds.

The clinical trial for Alzheimer's disease attaining most interest in recent years is the immunisation protocol performed with $A\beta_{1-42}$ by Elan Pharmaceuticals. Briefly, PDAPP transgenic mice were immunised with $A\beta_{1-42}$ and then showed a clearance of amyloid plaques (70). The investigations were rapidly progressed to immunisation of Alzheimer's disease patients. However, the study had to be terminated prematurely after a subgroup of the patients developed encephalitis. In the few post mortem studies available of patients that had been immunised, a low

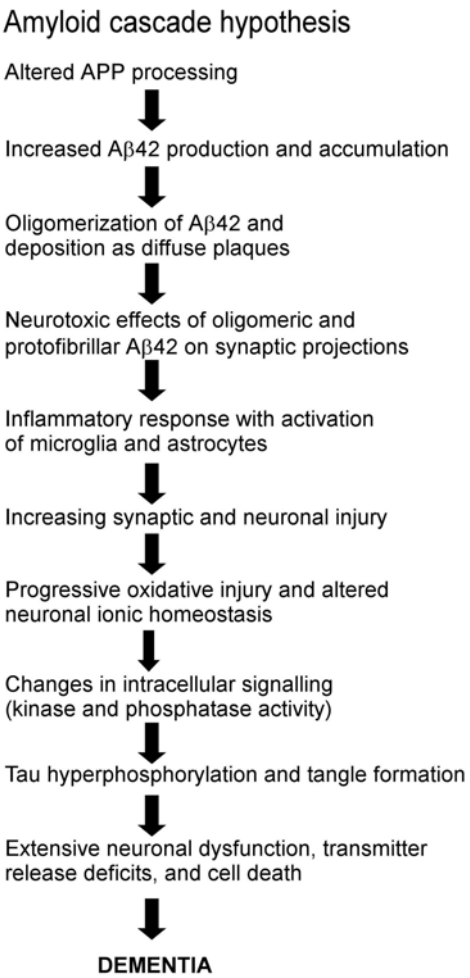

Figure 2. Outline of the amyloid cascade hypothesis illustrating the events leading to Alzheimer's disease (adopted from (5)).

amount of amyloid deposition, compared to the amounts seen in non-treated patients, could be detected indicating that $A\beta_{1-42}$ immunisation in humans could have an effect on plaque deposition (71). It has also been reported that immunised patients showed slower cognitive decline than non-treated patients (72). A drug that clears Aβ plaques is intuitively appealing, although the possibility that such clearance generates toxic Aβ oligomers should perhaps not be neglected. The severe side effects encountered in the initial clinical trial are of course not acceptable, but it is envisioned that side-effects can be attenuated by altering the immunogen (73). Likewise in clinical trials, and under preclinical development, are anti-Aβ antibodies administrated by passive immunisation. This is supposed to avoid a strong immunoreaction such as that generated by active Aβ immunisation. Proposed modes of action of administered Aβ antibodies include acting as a peripheral sink, whereby antibodies in plasma sequester Aβ from the brain, or that the antibodies can pass the blood brain barrier, and exert their activity directly in the brain (74).

The strategy of inhibiting Aβ aggregation using small molecules is principally attractive, considering their lower cost and easier administration compared to antibodies. This approach is, however, complicated by several potential obstacles. Preventing Aβ aggregation either by blocking intermolecular interactions necessary for polymerisation, or by stabilising the "native" structure of Aβ would potentially stop the pathogenic process before any toxic species could form. However, since there is no consensus as to what aggregates are the most toxic, finding out what step on the aggregation pathway to target is an unresolved key issue. Moreover, the structural flexible nature of soluble, monomeric Aβ (Figure. 3), and possibly also of Aβ oligomers, probably makes it difficult to find ligands that bind strongly to one particular structure. An alternative to reducing Aβ aggregation, at least theoretically, is to accelerate Aβ oligomer conversion to mature fibrils, which are supposed to be less cytotoxic. This approach could be truly biomimetic in the sense that fibrils have been suggested to represent one of the body's natural ways of sequestering toxic oligomers, and that ultra fast fibrillation is associated with formation of functional amyloid (75).

A large number of diverse compounds, including peptides and small organic molecules, have been shown to interfere with Aβ aggregation *in vitro*. Early studies concentrated on finding molecules that would prevent fibril formation, since the fibrils were believed to be the causative agent. However, when the idea of toxic oligomers surfaced, other compounds that could inhibit aggregation into early stages were presented. Examples of compounds identified as inhibitors of aggregation, irrespective of mode of action, include congo red, nicotine, scyllo-inositol, melatonin, rifampicin, curcumin, indole derivatives, methylene blue, □-cyclodextrin, hexadecyl-N-methylpiperidinium bromide and hemin (48, 66, 76-85).

Some of these compounds have also been shown to reduce Aβ-mediated cellular toxicity (77, 81, 83-88). Even though a number of these molecules share aromatic and/or hydrophobic features, there is no strict structural similarity between the small organic compounds that have all been shown to prevent Aβ aggregation. Therefore, they likely target different regions of the Aβ peptide and/or different forms of Aβ assemblies. The structures of some of these compounds are shown in Figure. 4 and points to the diversity of the group.

Necula *et al.* (89) recently studied a number of reported anti-Aβ aggregation compounds with the aim to determine if they acted as fibrillation or oligomerisation inhibitors, or both. Using an oligomer specific antibody (90) and turbidity/Thioflavin T fluorescence for detection of oligomer and fibril formation, respectively, the tested compounds fell into one of three groups; inhibitors of oligomer or fibril formation, or both. Some alkylammonium bromide surfactants ((Figure 4, (5)) showed biphasic behaviour; inhibition of fibril formation at low concentration and promotion of fibril formation at high. A biphasic behaviour for these compounds was reported previously (79), but then the effects were opposite; promotion of fibril formation at low concentrations and retardation at high concentrations. The main conclusion of the Necula *et al* study was that oligomer formation and

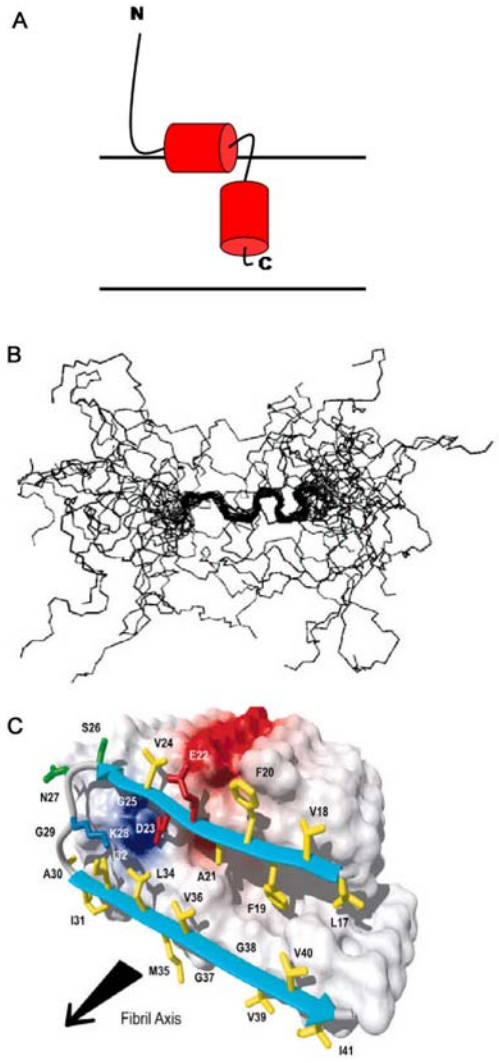

Figure 3. Structures of Aβ under different conditions. (A) Hypothetical structure of Aβ in a phospholipid membrane (illustrated by the two horizontal lines), just after its release from APP by β- and γ-secretase action (cf Figure. 1). The two barrels represent helical regions covering residues ~15–24 and ~29–C-terminus, respectively. The N-terminal helix is supposedly positioned superficially so that it can interact both with the hydrophilic surrounding and the hydrophobic membrane interior, while the C-terminal helix is buried in the hydrophobic part of the membrane. (B) Structure of $A\beta_{1-40}$ in water, represented by an ensemble of 20 calculated structures from NMR derived constraints. The structures are superpositioned in the region 16–24, which shows that this part possesses non-random structure. From (37) with permission from the publisher. (C) Structure of $A\beta_{1-42}$ fibrils. The region from residue 17 to 41 is shown, Leu17–Gly25 form an upper layer of parallel β-sheet and Ile31–Ile41 form a lower layer, and the intervening residues form a turn. The direction of the fibril axis is marked with an arrow. From (41) (copyright (2005) National Academy of Sciences, USA) with permission from the publisher.

fibril formation are the results of alternative pathways. The compounds were suggested to act by stabilising intermediates on either or both of these pathways (79).

Another cationic surfactant, hexadecyl-n-methylpiperidinium bromide (Figure 4, (3)), was found to inhibit $A\beta_{1-40}$ aggregation and fibril formation at concentrations well below its critical micelle concentration (82). The effects were concluded to be selective since small chemical modifications of the surfactant abolished the effects on Aβ, and since the aggregation of other amyloidogenic polypeptides (transthyretin and islet amyloid polypeptide) were not affected. The authors suggested that the prevention of Aβ aggregation could be mediated by binding of the amphiphile to the Aβ surface.

Yang *et al* (83) found that curcumin (Figure 4, (10)), which is a diphenolic compound with gross chemical similarities to Congo red (Figure 4, (1)) as well as to RS-0406 (Figure 4, (7)) (see below), inhibits $A\beta_{1-40}$ aggregation (as detected by Aβ antibody 6E10/6E10 ELISA) and fibril formation (by electron microscopy), and promotes Aβ disaggregation and dissolution of fibrils. Moreover, curcumin inhibited formation of $A\beta_{1-40}$ oligomers (tetramers and higher order oligomers, ~60kDa by SDS-PAGE), reduced $A\beta_{1-42}$ toxicity to N2a cells, and finally suppressed amyloid accumulation in Tg2576 transgenic mice after oral administration. According to another report curcumin does not inhibit fibril formation, but inhibits oligomer formation (89).

Screening a chemical library consisting of >100.000 compounds Nakagami *et al* (91, 92) found that RS-0406, a hydroxylated derivative of aniline (amino benzene), inhibits $A\beta_{1-42}$ fibril formation, protects neurons against $A\beta_{1-42}$-induced toxicity and reverses $A\beta_{1-42}$-induced impairment of long-term potentiation in hippocampal slices *in vitro*. Likewise, a similar compound, RS-0466, significantly inhibited Aβ-amyloid-induced cytotoxicity in HeLa cells and reversed the Aβ-amyloid-induced impairment of long-term potentiation in rat hippocampal slices. Using a cell line transgenic for APP that secretes Aβ oligomers (7PA2 cells (68)), it was found that these compounds inhibit the intracellular formation of Aβ oligomers (di- and trimers) (93). Moreover inhibition of long term potentiation by conditioned media from these cells was prevented by incubating the cells with RS-0406 or RS-0466, but not by incubating them with peptides based on the $A\beta_{16-20}$ (KLVFF) motif, in spite of the fact that these peptides inhibit $A\beta_{1-42}$ fibril formation.

From observations that Aβ oligomer and fibril formation is affected by phosphatidylinositol species (94-99), McLaurin and co-workers investigated different inositol isomers and found that scyllo-inositol (Figure 4, (4), also known as AZD-103) stabilises β-sheet structure in $A\beta_{1-42}$ and prevents $A\beta_{1-42}$ fibril formation. Interestingly, none of these effects were found for $A\beta_{1-40}$, although the toxicity of both $A\beta_{1-40}$ and $A\beta_{1-42}$ towards PC12 cells was reduced by scyllo-inositol (85). Scyllo-inositol was also

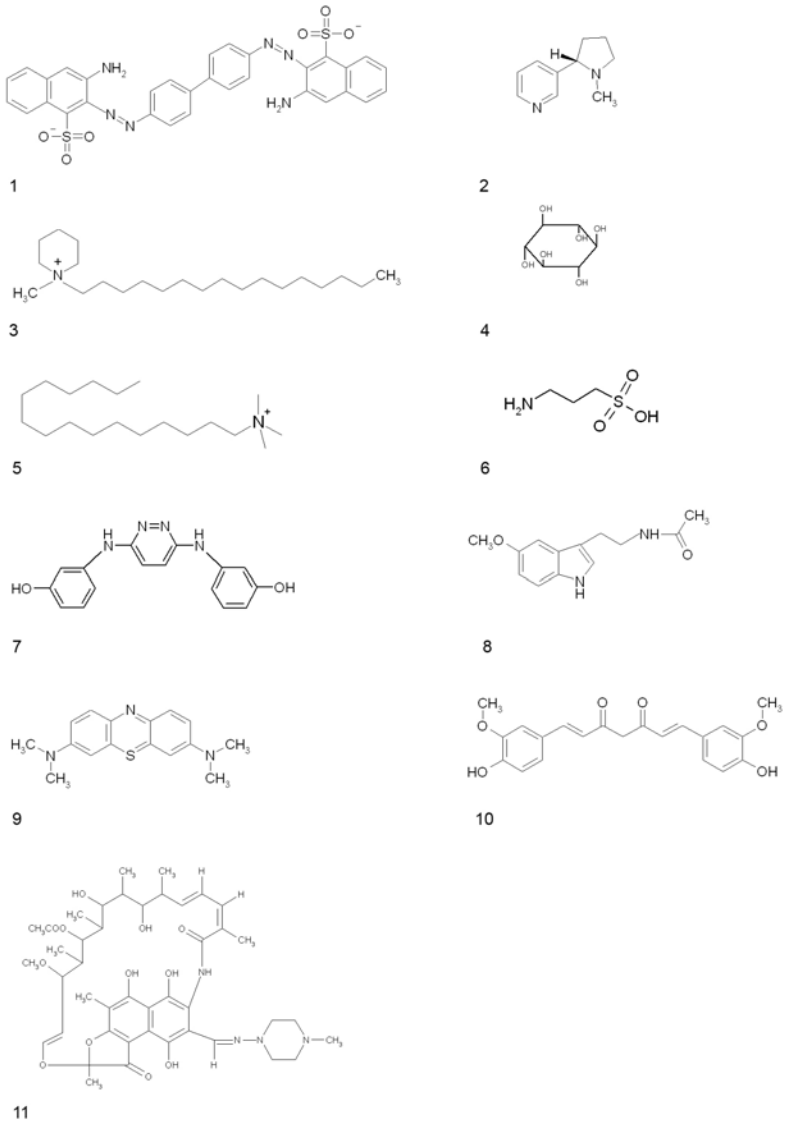

Figure 4. Chemical structures of low molecular weight compounds shown to interfere with the aggregation of Aβ. (1) congo red, (2) nicotine, (3) hexadecyl-n-methylpiperidinium bromide, (4) scyllo-inositol, (5) hexadecyl-triammonium, (6) amino-propane-sulfonic acid , (7) RS-0406, (8) melatonin, (9) methylene blue, (10) curcumin, (11) rifampicin. The structures are not drawn to scale.

found to reduce cognitive decline (using a Morris water maze memory test), reduce total amyloid plaque area as well as levels of soluble and non-soluble $A\beta_{1\text{-}40/42}$ in TgCRND-8 transgenic mice. These effects were seen irrespective of whether the treatment was started before or after onset of Alzheimer disease-like phenotype (100). Scyllo-inositol was found to rescue inhibition of long term potentiation by cell-derived soluble oligomers (101). The same authors also found that administration of scyllo-inositol to rats *via* the drinking water prevented interference of learned performance by cerebrovascular injection of cell-derived oligomers. Scyllo-inositol was found not to interfere with the production of Aβ low n-oligomers by 7PA2 cells, but to bind to Aβ dimers and trimers. It was suggested that scyllo-inositol works by binding to Aβ oligomers, preferentially trimers, and thereby masking epitopes that are important for their biological effects (101). McLaurin *et al* found that scyllo-inositol reduces the amount of high-molecular weight (~140 kDa) $A\beta_{1\text{-}42}$ and increases the amounts of trimers, and suggested that one possible mechanism of action of scyllo-inositol could be through inhibition of formation or disaggregation of high molecular weight Aβ oligomers (100).

Amino-propane-sulfonic acid, also known as tramiprosate, differs from a structural point of view from most other described Aβ aggregation inhibitors (Figure. 4, (6)). It also differs by its expected mode of action. It was designed to inhibit Aβ interactions with sulphated glycosaminoglycans and thereby prevent their ability to

promote Aβ structural conversion and fibril formation and stability (102). Amino-propane-sulfonic acid was found to maintain $Aβ_{1-40}$ in a non-fibrillogenic (random coil) form, to decrease $Aβ_{1-42}$ toxicity *in vitro*, and treatment of TgCRND-8 transgenic mice with amino-propane-sulfonic acid reduced cerebral amyloid plaque burden as well as levels of soluble and non-soluble $Aβ_{1-40/42}$ (23).

Considering their low degree of structural complexity the above mentioned compounds may suffer from a potential lack of specificity in binding to Aβ. One way to address this problem and to target a specific part of the Aβ peptides has been the development of peptide-based inhibitors, sometimes referred to as β-sheet breakers. The idea behind this approach is that a peptide could bind in a sequence-specific manner to a key region, necessary for Aβ-Aβ interaction, and thereby inhibit polymerisation and/or fibril formation. The first report based on this concept was by Tjernberg *et al.* who showed that the KLVFF motif ($Aβ_{16-20}$) acts as an inhibitor of full length Aβ aggregation, and that it is actually sufficient for forming amyloid fibrils on its own (103). Soto *et al.* developed a β-sheet breaker molecule based on the region Leu17-Ala21 with proline-substitution, to interfere with backbone hydrogen-bonding and to reduce the β-sheet propensity of the ligand itself (104). One of these ligands (LPFFD), was shown to be stable *in vivo*, to penetrate the blood-brain barrier, and to clear amyloid plaques in a rat model (105, 106). Several groups have studied the effects of introducing N-methylated amino acids in small peptide inhibitors, thereby creating a "blocked" side of the ligand that should effectively halt fibril elongation by blocking inter-strand hydrogen bonding (107-109). Recently, Arvidsson and co-workers determined the first structure at atomic resolution of a fully N-methylated peptide by X-ray crystallography, which showed that it adopts an elongated, β-strand conformation and that the backbone carbonyl groups are positioned such that they can hydrogen bond to another peptide in a β-strand conformation (110).

Very recently an illuminating study of fibrillation inhibitors, including some inhibitors of Aβ aggregation, was published (111). The main finding presented was that compounds that are known to form colloidal aggregates, *e.g.* Congo red, inhibit fibril formation and *vice versa*, several known fibrillation inhibitors were found to act as aggregators. The discomforting conclusion was that chemical aggregators are common among inhibitors of amyloid fibril formation. Chemical aggregators form colloid that may physically sequester proteins in a promiscuous and non-specific manner and they can thereby also interfere with fibril formation (112, 113). Colloid-forming compounds are typically highly conjugated, hydrophobic and dye-like (111), features which are common among published Aβ aggregation inhibitors (Figure. 4). With the data of Shoichet and coworkers in hand, it seems that the mechanism of action of several Aβ aggregation inhibitors may need to be revised, and that future screens for such inhibitors have to exclude compounds that form chemical aggregates (111, 114).

7. CONCLUSIONS AND PERSPECTIVES

Developing strategies for inhibiting Aβ aggregation *in vitro* is possible using a wide range of interacting molecules as illustrated by the examples above. However, underlying mechanisms of action need to be carefully investigated to exclude non-specific actions. Moreover, whether these results transfer to humans and will actually have a beneficial effect in patients suffering from Alzheimer's disease is not clear yet.

Better understanding of the aggregation process and the disease causing assemblies *in vivo* is crucial for the development of potent therapeutic strategies. An important step in the search for inhibitors is to develop assays that can clearly distinguish between the different intermediate species of the aggregation pathway. This would allow for easier screening of compounds and targeting of the right aggregation intermediate. It is important to bear in mind that when the first symptoms of the disease become evident, with reduced cognitive abilities, it is the result of a neurodegenerative process that has likely been ongoing for many years. The starting point is the generation and accumulation of the Aβ peptide and the end stage is the atrophic brain containing amyloid plaques. Attaining an idea of the correct order of events in the disease development is challenging since it is an ongoing process with different assembly states occurring simultaneously. The aim of identifying "the" toxic species in humans is a desirable, but tough, goal with all the different intermediate assemblies being present. The methods used to resolve which Aβ species are present in *in vitro* and *in vivo* studies are often harsh since aggregated Aβ will not be easily dissolved or extracted. Hence, the possibility that artefacts can be introduced during experimental handling should not be neglected. One approach that has recently become available is the use of conformational specific antibodies (90). However, the exact epitope of this type of antibody is not defined. The research field would benefit much if the methods used for isolation of Aβ aggregates of varying size as well as the nomenclature were to be more stringently defined.

There are today many powerful techniques available for molecular studies of Aβ. For example, NMR can give us the exact structure of monomeric as well as fibrillar Aβ. Nevertheless, it is uncertain to what extent these structures represent the Aβ forms that are produced in the Alzheimer's disease brain. It is of great importance to characterise the naturally occurring Aβ derived from human brain to enable studies of synthetic preparations which will give us physiologically relevant information. For example, by using primary cultured neurons with synaptic contacts together with the appropriate Aβ species we will get biologically important information. In the years to come we will hopefully see a transition were studies are performed with an even closer proximity between *in vitro* and *in vivo* results.

At present, many studies suggest that some sort of soluble oligomeric form of Aβ results in a synaptotoxic effect. Hence, the focus on studying pre-fibrillar species will continue and further deepen our understanding of the molecular structures and events underlying Alzheimer's disease. This greater knowledge will aid in designing new therapeutic strategies and evaluate existing pharmacological appraoches, i.e. in one way or another prevent oligomer formation. The broad spectrum of potential therapeutic strategies from enzyme inhibition and small molecular interference of aggregation to immunisation, together with a devoted field of researchers

holds promise for progress in basic understanding of the disease and in drug development.

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Abbreviations: Aβ, amyloid β-peptide; ADDL, Aβ-derived diffusible ligands; APP, amyloid precursor protein.

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