Chapter 18

Amyloid Inhibitors and β-Sheet Breakers

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Abstract: Compelling evidence indicates that a key pathological event in Alzheimer's disease is the misfolding and aggregation of normal soluble amyloid-β peptide into β-sheet-rich oligomeric structures which have a neurotoxic activity and ability to form insoluble amyloid deposits that accumulate in the brain. β-sheet breakers constitute a new class of drugs that are designed to specifically bind amyloid-β peptide blocking and/or reversing the misfolding process. In this article we review this approach and summarize the data supporting the view that β-sheet breakers could be serious candidates to combat this devastating disease.

Key words: Alzheimer's disease; amyloid; β-sheet breakers; therapy

1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of late-life dementia, and represents one of the leading causes of death in the developed world. AD is a progressive and devastating neurodegenerative disorder characterized by memory loss, and a variety of cognitive disabilities. This disease is a major social and health care problem, because of its increasing prevalence, long duration and high cost of care. Despite important efforts aimed at elucidating its underlying molecular mechanisms, there is still no effective treatment available for this disease.

The major neuropathological changes in the brains of AD patients are neuronal death, particularly in regions related to memory and cognition and the presence of abnormal intra- and extracellular abnormal protein aggregates, known as neurofibrillary tangles and amyloid plaques, respectively (Terry, 1994; Selkoe, 1997). Tangles are paired helical filaments composed largely of abnormally hyperphosphorylated tau protein (Terry, 1994). Amyloid plaques deposit in AD brain parenchyma in the form of senile plaques and around the cerebral vessels walls (Selkoe, 1997). Amyloid deposits have revealed to be essentially composed of aggregated amyloid-β peptide (Aβ). This 39-42 residue peptide is derived from the processing of a large ubiquitous type I transmembrane protein called APP (amyloid precursor protein), whose gene is located on chromosome 21 (Selkoe, 1997). The Aβ domain, which starts on the cell surface and ends within the membrane is sequentially cleaved by β- and γ-secretase at the N- and C-terminus, respectively (Selkoe, 1997). Although the role of tangles and amyloid plaques in the pathogenesis of the disease is not fully understood they represent a typical signature of AD and their presence is needed to make a definitive postmortem diagnosis of the disease.

2. IS AMYLOID THE CAUSE OF AD PATHOGENESIS?

In spite of numerous studies aimed at understanding the aetiology of AD, the triggering events leading to neuropathology remain to be clearly defined. Compelling evidence has accumulated in the last fifteen years indicating that amyloid may be the cause of neurodegeneration in AD or at least a central event in the pathogenesis (Selkoe, 1997; Selkoe, 2000b). These evidences come primarily from studies of individuals affected by the familial form of the disease or by Down's syndrome patients. Almost all patients with Down's syndrome (trisomy 21), which contain three copies of the APP gene, develop AD neuropathology at an early age, and the presence of diffuse amyloid deposits has been shown to precede other alterations (Mann, 1989). The fact that mutations in the amyloid protein precursor (APP) gene are associated to familial AD is a strong indication of the importance of amyloid in the pathogenesis of the disease (Selkoe, 2000a; Hardy *et al.*, 1998). In addition to *APP*, three other genes have been so far linked with AD: apolipoprotein E (*apoE*, chromosome 19), presenilin 1 (*PS1*, chromosome 14) and presenilin 2 (*PS2*, chromosome 1). Mutations or polymorphisms in these genes induce an increase in the production or amyloidogenicity of Aβ and therefore appear to be implicated in the disease through the formation of amyloid (Selkoe, 2000a; Selkoe, 2000b; Younkin, 1995; Fraser *et al.*, 2000; Price *et al.*, 1998). Moreover, transgenic mice that express high levels of

human mutant APP progressively develop many of the pathological hallmarks of AD, including cerebral amyloid deposits, neuritic dystrophy, astrogliosis and behavioral alterations (Price *et al.*, 1998; Van Leuven, 2000).

Based on these findings it was thought during several years that mature amyloid plaques deposited in brain parenchyma were the culprit of neurodegeneration and disease. This idea was further supported by results *indicating* that amyloid aggregates are toxic to neuronal cells in culture and that citotoxicity was dependent upon Aβ aggregation (Pike *et al.*, 1993; Lorenzo and Yankner, 1994). However, in the last couple of years this view has been changing progressively in favour of soluble misfolded oligomeric intermediates as the toxic specie (Caughey and Lansbury, 2003; Walsh *et al.*, 2002). Recent in vitro experiments obtained in cell culture experiments suggest that the soluble oligomeric intermediates (also known as Aβ-derived diffusible ligands) and protofibrils could be even more toxic than amyloid fibrils (Walsh *et al.*, 1999; Lambert *et al.*, 1998). Moreover, temporal studies of the appearance of AD-like alterations in APP transgenic animals have shown that significant cerebral damage and cognitive impairment appear before amyloid plaque detection (Moechars *et al.*, 1999). The nature of the toxic Aβ specie is very important to design efficient therapeutic strategies based on arresting the negative influence of the Aβ pathway. Indeed, some scientists have proposed that the deposition of amyloid plaques could even be considered a protective event allowing the deposition and isolation of the toxic abnormally folded Aβ oligomers (Caughey and Lansbury, 2003).

A clear mechanism to explain the role of Aβ in neurodegeneration is still missing (Yankner, 1996; Soto, 2003). Cell culture experiments involving the conversion of soluble Aβ into β-sheet rich Aβ have suggested that amyloid deposits, protofibrils and/or soluble oligomers could be directly toxic to neurons (Selkoe, 1997; Soto, 2003; Caughey and Lansbury, 2003). Various mechanisms have been proposed to explain Aβ neurotoxicity, including mitochondrial redox activity impairment leading to increased free radicals, intracellular Ca^{2+} increase, ion channels formation, or signal transduction by interaction with specific cellular receptors (Soto, 2003; Yankner, 1996). Alternatively, amyloid could be indirectly toxic through a locally induced chronic inflammatory response, with the abnormal Aβ structures acting as an irritant that triggers inflammation (Soto, 2003; McGeer and McGeer, 1998). Indeed, AD brains are characterized by microglia and astrocyte activation, as well as by numerous inflammatory proteins, including complement factors, pro-inflammatory cytokines and acute phase-reactant proteins (McGeer and McGeer, 1998). However, it remains to be clarified whether one central mechanism mediates amyloid toxicity or if it is dependent on an interplay of several direct and indirect processes.

3. AMYLOID AGGREGATION INHIBITORS

Aβ misfolding and aggregation is probably the first pathological processes in AD. This fact and the abundant knowledge accumulated about the molecular mechanism of amyloid formation make the inhibition of Aβ misfolding and oligomerization an attractive therapeutic target for AD. However, uncertainties respect to which Aβ species is mostly involved in the disease pathogenesis complicate this approach, because inhibiting the process in the wrong step may lead to accumulation of toxic intermediates.

Several unrelated small molecules have been shown to prevent and/or reverse Aβ polymerization *in vitro*. Among these compounds is possible to mention the following (Fig. 1): congo red (Lorenzo and Yankner, 1994), hexadecyl-N-methylpiperidinium bromide (Wood *et al.*, 1996), small sulfonated anions (Kisilevsky *et al.*, 1995), benzofuran-based compounds (Allsop *et al.*, 2001), rifampicin (Tomiyama *et al.*, 1994), melatonin (Pappolla *et al.*, 1998), nicotine (Salomon *et al.*, 1996), estrogen (Hosoda *et al.*, 2001), glycosaminoglycans mimetics (Gervais *et al.*, 2001), nitrophenols (De Felice *et al.*, 2001), tetracycline (Forloni *et al.*, 2001), anthracycline 4'-iodo-4'-deoxydoxorubicin (Merlini *et al.*, 1995), clioquinol (Cherny *et al.*, 2001), ibuprofen (Lim *et al.*, 2000) and N,N'-bis(3-hydroxyphenyl)pyridazine-3,6-diamine (Nakagami *et al.*, 2002). The activity of many of these compounds has also been shown in vivo using AD animal models and even some of them are currently under clinical evaluation in AD patients. However, the usefulness of these small molecules as amyloid inhibitors is compromised by their lack of specificity and their (in most of the cases) unknown mechanism of action, which makes it difficult to improve them. In addition, many of these compounds are highly toxic.

A more rational approach to make inhibitors of amyloid formation is to design specific peptide ligands based on the well-known self-recognition ability of Aβ and on the study of the structural requirements for Aβ aggregation (Soto, 1999; Findeis, 2002; Mason *et al.*, 2003). This knowledge was used to develop peptide inhibitors that contain the self-recognition motif, which binds Aβ and partially inhibits amyloid fibril formation *in vitro*. Several different peptides around the sequence 16-22 of Aβ are under development (Soto, 1999; Findeis, 2002; Mason *et al.*, 2003).

Tjernberg and co-workers showed that the Aβ(16–20) peptide is able to bind full-length Aβ and prevent its assembly into fibrils (Tjernberg *et al.*, 1996).

Figure 1

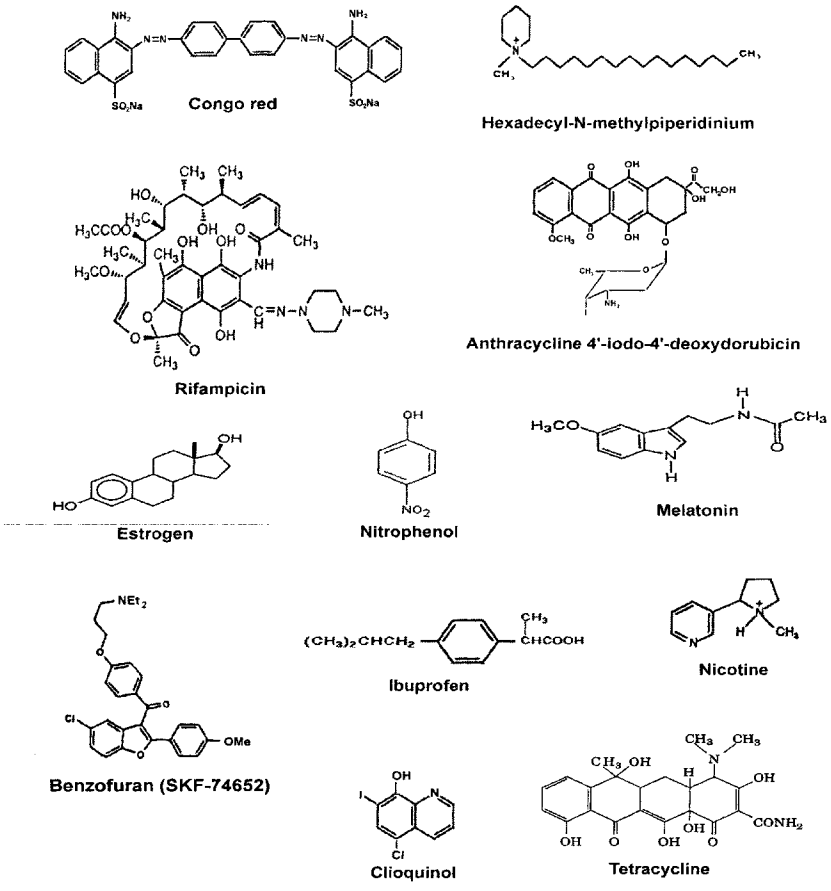

Figure 1. Chemical structure of some small-molecule amyloid inhibitors. Several structurally diverse small chemical molecules have been reported to inhibit either amyloid fibril formation or amyloid neurotoxicity in vitro. Some of them have also been shown active in animal models of AD. Unfortunately, their lack of specificity and, in many cases, toxicity diminishes their therapeutic use.

Using molecular graphics simulations, they hypothesized that it binds stereospecifically and in an antiparallel conformation to Aβ. However, Aβ (16–20) spontaneously aggregates into amyloid-like fibrils and thus its use

as an inhibitor might be problematic. Therefore, several groups began to modify this sequence to produce peptide derivatives containing the self-recognition motif, but at the same time a disrupting element enhancing their inhibitory activity. Our approach using β-sheet breaking amino acids was the first to lead to modified peptides with inhibitory activity (Soto *et al.*, 1996). This strategy will be discussed in the next section.

Based on the fact that the major force driving Aβ aggregation is hydrophobicity, Murphy and colleagues have added charged residues to the ends of the recognition motif as a disrupting element (Ghanta *et al.*, 1996; Pallitto *et al.*, 1999). Having shown that at least three lysines are required as an appropriate disrupting element, the compound (KLVFFKKKK) showed activity in altering fibril morphology and reducing cellular toxicity in vitro. The anionic disrupting compound KLVFFEEEE had similar effects, whereas the neutral compound KLVFFSSSS was ineffective, suggesting that the charged nature of the disrupting element is critical (Pallitto *et al.*, 1999). Another approach to produce peptide inhibitors have been reported by Findeis and co-workers (Findeis *et al.*, 1999; Findeis *et al.*, 2001). Their strategy was to retain a peptide sequence that could bind to Aβ and add a bulky group, such as a steroid, at its terminus to hinder Aβ polymerization. The all-D-amino acid peptide cholyl-LVFFA-OH was shown to be a potent inhibitor of Aβ polymerization, but was cleared up almost completely upon hepatic first pass, possibly because the cholyl group was recognized as an endogenous bile component (Findeis *et al.*, 2001). Several other peptides with different chemical groups having improved pharmacological properties are under development. Various teams are studying the incorporation of *N*-methyl amino acids into peptides as disrupting elements (Hughes *et al.*, 2000; Gordon *et al.*, 2001). The idea behind is that one side presents a hydrogen-bonding 'complementary' face to the protein, with the other side having *N*-methyl groups in place of backbone NH groups, thus presenting a 'blocking' face (Mason *et al.*, 2003). Hughes *et al.* have shown that *N*-methyl derivatives of Aβ(25–35) are able to prevent aggregation and inhibit toxicity in PC-12 cells (Hughes *et al.*, 2000). Meredith and co-workers investigated *N*-methylated peptides corresponding to 16–22 and subsequently 16–20 sequence of Aβ. These peptides can prevent Aβ fibrils from forming and break down preformed fibrils (Gordon *et al.*, 2001). N-methylation contributes additionally to make the peptides more resistant to proteolysis than regular peptides (Adessi and Soto, 2002). More recently, Kapurniotu and colleagues reported an Aβ1-28 analog constrained by an internal cycle between residues Lys17 and Ala21. This modified peptide inhibited Aβ aggregation and cytotoxicity (Kapurniotu *et al.*, 2003).

4. β-SHEET BREAKERS

β-sheet breakers have emerged in the last few years as the prototype class of compounds inhibiting and reversing protein misfolding and aggregation. Indeed, although we coined this name to refer specifically to our compounds rationally-designed to break β-sheets, in recent years the β-sheet breaker concept has been used to refer to any compound with amyloid inhibitory properties, such as some of those described in the previous section.

Our approach to produce β-sheet breakers uses the Aβ self-recognition motif (17-20) to achieve binding and specificity, but replacing a residue

Figure 2

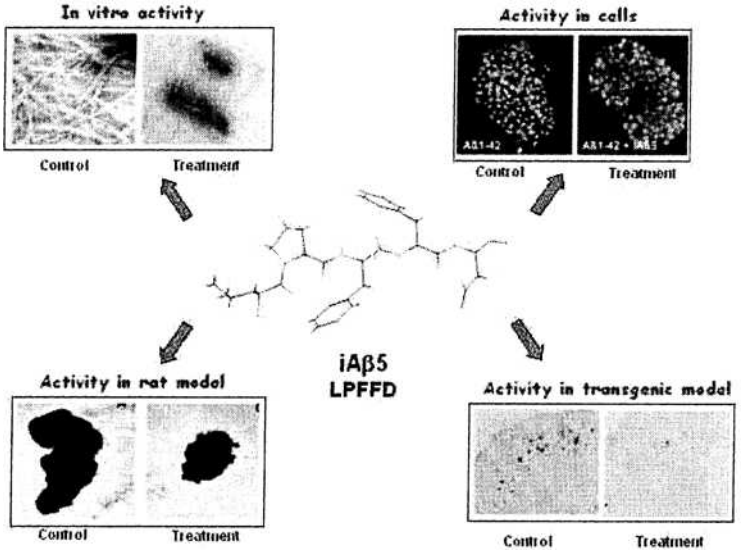

Figure 2. β-sheet breaker peptides as a promising approach for the treatment of AD. A 5 residues peptide (chemical structure in center panel) has been shown to be active in several in vitro, cellular and in vivo models. In vitro studies showed that the compound was able to inhibit and dissolve amyloid plaques in the test tube, as studied by electron microscopy (top, left panel) among other techniques. In cell cultures, the peptide prevented neuronal death induced by amyloid (top, right panel) as studied by staining procedures that allow to distinguish healthy (green) from death (orange) cells. In vivo experiments were done by using two different animal models; a rat model of cerebral amyloidosis (bottom, left panel) and a double transgenic mice model of AD (bottom, right panel). In both models, the 5 residues b-sheet breaker showed a significant activity on preventing and dissolving amyloid plaques.

important for forming β-sheets by an amino acid thermodynamically unable to fit inside this structure (Soto *et al.*, 1996; Soto, 1999). Valine at position 18 of Aβ plays an important role on stabilizing β-sheet folding in Aβ (Soto *et al.*, 1995), but seems not to be absolutely necessary for self-recognition. Therefore, we decided to replace this amino acid by proline, a residue that because of its particular chemical structure is an efficient β-sheet breaker (Wood *et al.*, 1995; Kim and Berg, 1993). A prototype 5-residue β-sheet breaker peptide (iAβ5 Seq: LPFFD) has been tested in different in vitro, cellular and *in vivo* assays (Figure 2). The *in vitro* activity was quantitatively demonstrated using a thioflavine T binding assay, which is a commonly used fluorometric method to quantify amyloid, and qualitatively confirmed by electron microscopic examination of amyloid fibrils (Soto *et al.*, 1996; Soto *et al.*, 1998). This peptide appeared not only to inhibit the abnormal Aβ conversion and subsequent amyloid formation, but also to dissolve preformed fibrils (Soto *et al.*, 1996; Soto *et al.*, 1998). Human neuroblastoma cell culture experiments revealed that iAβ5 was able to prevent neuronal death induced by the formation of β-sheet-rich oligomeric Aβ structures (Soto *et al.*, 1998). Two animal models have been employed to monitor the activity of β-sheet breaker peptides *in vivo*. In the first model, amyloid deposition was induced by injecting non-aggregated Aβ1-42 in rat brain. After some time, a single fibrillar lesion with ultrastructural properties similar to AD amyloid plaques could be observed at the site of injection together with some AD-typical neurodegenerative features such as extensive neuronal shrinkage, astrocytosis and microglial activation (Soto *et al.*, 1998; Sigurdsson *et al.*, 2000). Co-injecting iAβ5 with Aβ1-42 reduced cerebral Aβ accumulation and completely prevented the formation of fibrillar amyloid-like lesions (Soto *et al.*, 1998). In a second experiment in the rat model, iAβ5 even proved to be able to induce a significant reduction in the size of preformed Aβ fibrils when it was injected into the cerebral amygdala 8 days after injecting Aβ at the same place (Sigurdsson *et al.*, 2000). A reversion of the associated cerebral histopathological changes, such as neuronal shrinkage and microglial activation, was also observed in this experiment. No effect was detected by injecting unrelated peptides (used as controls) under the same conditions.

5. FROM β-SHEET BREAKER PEPTIDES TO β-SHEET BREAKER DRUGS

Like most short unmodified peptides, β-sheet breaker peptides are very prone to peptidase degradation and exhibit very short half-lives *in vivo* (Adessi *et al.*, 2003; Adessi and Soto, 2002). For example, incubation of

iAβ5 in human plasma revealed a half-life of only 5 minutes (Permanne *et al.*, 2002a). Since no metabolites could be detected, it was concluded that degradation was complete and most probably due to exopeptidases. In order to block this enzymatic activity, iAβ5 was end-protected by N-terminal acetylation and C-terminal amidation, yielding iAβ5p (Ac-LPFFD-NH_2). iAβ5p appeared to be very stable both in human plasma and CSF, with little or no degradation after 24 hours of incubation (Permanne *et al.*, 2002a). This better stability made possible the evaluation of iAβ5p efficacy in a more relevant transgenic animal model by reasonable routes of administration. A double transgenic mouse model overexpressing human APP with the London mutation (V717I) and human PS1 with the A246E mutation develops many AD pathological features, such as extensive deposition of amyloid plaques, neuritic dystrophy, astrogliosis and some degree of tau neuropathology (Dewachter *et al.*, 2000). Such animals were treated with iAβ5p via intracerebroventricular infusion (icv) or intraperitoneal injection (ip, three times a week) for a period of 8 weeks. This treatment resulted in a significant lowering of cerebral amyloid load (67.3% and 46% for icv and ip, respectively) compared to vehicle administration, as well as a decreased brain inflammation and neuronal loss (Permanne *et al.*, 2002b). However, by contrast with *in vitro* stability experiments, pharmacokinetic studies in rats revealed an *in vivo* iAβ5p half-life of only 37 minutes. In order to further increase enzymatic stability, we identified the major cleavage sites in vivo and protected these peptide bonds by introducing various chemical modifications around them (Adessi *et al.*, 2003). From the numerous newly engineered iAβ5p derivatives, one peptide appeared to have the same *in vitro* activity as iAβ5p in inhibiting amyloid fibril formation and amyloid neurotoxicity, while exhibiting a much higher stability in rat brain homogenate and a 10-fold greater half-life after intravenous administration to mice (Adessi *et al.*, 2003).

Minimizing the weakness of a peptide by specific chemical modifications may increase the drug-like properties of the compound, but in order to have an orally available and highly effective drug, the design of a non-peptide mimetic is needed. For this purpose, it is important to know the mechanism of action of the lead compound, the chemical groups responsible for activity and the tridimensional structure of the active compound (Adessi and Soto, 2002; Adessi and Soto, 2002; Moore, 1994).

Although numerous experiments involving various β-sheet breaker peptides have shown that these drug candidates were very efficient both *in vitro* and *in vivo*, the mechanism underlying their activity remains mostly unclear. β-sheet breaker peptides appear to bind to the central self-recognition motif of Aβ through hydrophobic interactions. Hydrophobicity is also the major driving force for Aβ-Aβ interaction through the self-recognition sequence (Soto *et al.*, 1994). Indeed, the replacement of

hydrophobic residues by hydrophilic ones in this region of Aβ has been shown to prevent amyloid formation, thereby suggesting that aggregation of Aβ monomers is driven by hydrophobic interactions (Hilbich *et al.*, 1992). Since β-sheet breaker peptides exhibit a certain degree of homology to the self-recognition region of Aβ, they may compete with Aβ monomers to bind this region through similar interactions. Consistent with this view, molecular modelling studies of the interaction between the three-dimensional structure of Aβ1-40 and iAβ5p, both elucidated by nuclear magnetic resonance, suggest that the β-sheet breaker binds to a hydrophobic pocket in the central region of Aβ in an antiparallel way (unpublished observations).

From our structure-activity studies, it seems that Pro and Phe at positions 2 and 3, respectively, play a fundamental role in the activity of β-sheet breaker peptides (Adessi *et al.*, 2003). It has been shown previously by several groups that Phe 19 (equivalent to the Phe 3 of iAβ5p) is critical for Aβ-Aβ interaction and amyloid formation (Hilbich *et al.*, 1992; Tjernberg *et al.*, 1996; Wood *et al.*, 1995). It is likely that the phenylalanines (second and third position) and leucine (first position) residues are responsible for selective binding of the β-sheet breaker peptides to Aβ. Also an ionic interaction between positively and negatively charged residues in Aβ and iAβ5 appears to be important for binding affinity. On the other hand, the Pro residue does not seem to play a role on binding, but is critical for activity. These results suggest the importance of maintaining a key balance between the sequence homology between Aβ and the β-sheet breaker, and the presence of amino acids able to disrupt β-sheet folding. Indeed, a β-sheet breaker peptide with full sequence homology to Aβ would have a strong affinity but would not be able to block the pathological conformational change. On the other hand, a compound with too many proline residues to inhibit β-sheet structures would be inactive because of low affinity due to poor homology.

With all this information in hand, now the aim is to design compounds with similar chemical groups in the correct tridimensional position but tied together by a non-peptidic scaffold (Adessi and Soto, 2002; Moore, 1994). Although this is a challenging task, the small size of the peptide lead and the abundant knowledge of its structure and properties, coupled with the accelerated advance on scaffold chemistry, make it feasible.

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