# Synthesis and Biological Investigation of β-Amyloid Peptides

**PhD Thesis**

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## Contents

## List of publications related to the subject of the Thesis

**Full length papers**

I. Laskay G., Zarándi M., Varga J., **Jost K.**, Fónagy A., Torday C., Latzkovits L., Penke B. A putative tetrapeptide antagonist prevents β-amyloid-induced long-term elevation of $[Ca^{2+}]i$ in rat astrocytes.
*Biochemical and Biophysical Research Communications*, **235**, 479-481, 1997.
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II. Harkány T., Ábrahám I., Laskay G., Timmerman W., **Jost K.**, Zarándi M., Penke B., Nyakas C., Luiten P.G. Propionyl-IIGL tetrapeptide antagonizes beta-amyloid excitotoxicity in rat nucleus basalis.
*Neuroreport*, **10**, 1693-1698, 1999.
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III. Szabó Z., **Jost K.**, Soós K., Zarándi M., Kiss J.T., Penke B. Solvent effect on aggregational properties of β-amyloid polypeptides studied by FT-IR spectroscopy
*Journal of Molecular Structure,* **480**, 481-487, 1999.
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IV. Szabó Z., Klement E., **Jost K.**, Zarándi M., Soós K., Penke B. An FT-IR study of the β-amyloid conformation: standardization of aggregation grade.
*Biochemical and Biophysical Research Communications*, **265**, 297-300, 1999.
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V. Laskay G., Zarándi M., Jost K., Penke B., Bálint E., Ocsovszki I., Tarcsa M., Várszegi S., Gulya K. β-Amyloid[1-40]-induced early hyperpolarization in M26-1F cells, immortalized rat striatal cell line.
*Neurobiology,* 7, 431-436, 1999.
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VI. **Jost K.**, Varga J., Zarándi M., Penke B. *In vitro* degradation of β-amyloid[25-35] peptide. *Protein and Peptide Letters*, **8**, 423-428, 2001.
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Published abstracts

VII. **Jost K.**, Varga J., Szabó Z., Penke B., Zarándi M. Chemical degradation of β-amyloid[25-35] peptide under physiological conditions.
*Naunyn-Schmiedeberg's Archives of Pharmacology*, **356/4** Suppl.1: R36, 1997.

VIII. **Jost K.**, Varga J., Zarándi M., Penke B. Stability studies on β-amyloid[25-35] peptide.
*Peptides 1998. Proceedings of 25$^{th}$ European Peptide Symposium,* 724-725, 1998.

## Abbreviations

| | |
|---|---|
| Aβ | β-amyloid peptide |
| AD | Alzheimer's disease |
| All | allyl |
| apoE | apolipoprotein E |
| APP | amyloid precursor protein |
| Asu | aminosuccinimide |
| BBB | blood-brain barrier |
| Boc | *tert*-butyl-oxy-carbonyl |
| 2BrZ | 2-bromo-benzyl-oxy-carbonyl |
| $Bu^t$ | *tert*-butyl |
| Bzl | benzyl |
| $[Ca^{2+}]_i$ | intracellular $Ca^{2+}$ |
| CD | circular dichroism |
| cHex | cyclohexyl |
| 2ClZ | 2-chlorobenzyl-oxy-carbonyl |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| DCC | dicyclohexyl carbodiimide |
| DIEA | N,N-diisopropyl-ethyl-amine |
| DMF | dimethyl-formamide |
| DMSO | dimethyl sulfoxide |
| ESI | electrospray ionization |
| FAB | fast atom bombardment |
| FAD | familiar Alzheimer disease |
| Fm | fluorenyl-methyl |
| Fmoc | fluorenyl-methoxy-carbonyl |
| FT-IR | Fourier transform infrared spectroscopy |
| GABA | gamma-aminobutyric acid |
| HFIP | hexafluoroisopropanol |
| HOBt | 1-hydroxybenzotriazole |
| HPLC | high performance liquid chromatography |
| LC-MS | liquid chromatography combined with mass spectrometry |
| MBHA | 4-methyl-benzhydyl-amine |
| MS | mass spectrometry |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide |
| NFT | neurofibrillary tangels |
| NMDA | N-methyl-D-aspartate |
| O.D. | optical density |
| PBS | phosphate buffer solution |
| Ph | phenyl |
| Pr | propionyl |
| RP | reversed phased |
| sAPP | secreted or soluble APP |
| SPPS | solid phase peptide synthesis |
| Pmc | pentametil-cromane |
| TFA | trifluoroacetic acid |
| Tos | *para*-toluene-sulfonyl |
| Z | benxyloxycarbonyl |

# 1. Introduction and aims

Peptides have very important role in bioregulation as hormones, neurotransmitters, neuromodulators, etc. In the organism, biologically active peptides are present at very low concentrations (e.g. in $10^{-10}$ M in the blood plasma), and their metabolism are strictly regulated. If the concentration of a biologically active peptide increases in an uncontrolled manner, it can cause serious problems in the bioregulation. Under normal conditions, proteolytic enzymes cleave peptides very rapidly. However, in several cases, the overproduction or defective clearance of peptides causes serious illnesses. The brain is highly sensitive to long-lasting elevated concentrations of neurotransmitters or neuropeptides. It can be stated that some neuropeptides, which have important physiological roles, are neurotoxic in high concentrations. Alzheimer's disease (AD) is an example for the neurotoxicity of a peptide, β-amyloid peptide (Aβ).

Our aims were as follows:

1. to optimize the synthesis of Aβ peptides, peptide fragments and modified Aβ peptides
2. to investigate the biological effects of Aβ peptides in various biological assays
3. to design and synthesize peptides which inhibit the neurotoxic effect of Aβ (functional antagonists); to test them in cell culture
4. to investigate the stability of Aβ peptides under physiological conditions and to establish applicable peptide handling for *in vivo* and *in vitro* experiments.
5. to characterize the secondary structure and the aggregation state of Aβ peptides.

# 2. Theoretical background

## 2.1 Theories about Alzheimer's disease

There is a significant increase in the prevalence of disorders affecting elderly people, as more and more people reach old age. Few subjects in biomedicine have aroused the interest of the scientific and lay communities alike as has Alzheimer's disease (AD). The dramatic rise in life expectancy during the 20$^{th}$ century, from roughly 49 years to more than 75 years in the USA and countries of the western world, has resulted in a burgeoning number of individuals achieving the age of at which neurodegenerative disorders become common. The most common form of age-related neurodegenerative disorders is AD. It was first described by the German physician Alois Alzheimer in 1907 [1]. Multiple molecular, cellular, structural, and functional changes occur in the brain during aging. Neural cells may respond to these changes adaptively, or they may succumb to neurodegenerative cascades that result in disorders such as AD [2].

One of the major neuropathological characteristics of AD is the extracellular accumulation of β-amyloid peptides (Aβ) in neuritic plaques [3,4,5,6]. Aβ is normally secreted by neurons and can be found in low concentrations in cerebrospinal fluid (CSF) [7,8] and plasma where it is associated with lipoproteins [9,10,11]. However, the physiological role of Aβ secretion remains unknown [12,13]. Experimental data indicate that AD affects a wide array of neuronal and glial functions and thereby may lead to neuronal death in the nervous system. The fatal outcome of exogenous Aβ administration in numerous neurotoxicity models is well established, but the mechanism is still elusive.

Amyloid substances are tissue deposits occurring in animals and humans, primarily composed of unique proteinaceous fibrils. Deposition of the relatively inert fibrils by different pathogenic mechanisms leads to excessive accumulation, with pressure atrophy and death, resulting from interference with physiological processes of affected organs. This disease complex is referred to as amyloidosis or β-fibrilloses; it occurs in association with inflammatory conditions (such as rheumatoid arthritis), and with tumours of various types. Aging is thought to occur in association with amyloid deposition in the brain, heart and pancreas. Whereas amyloid variants exhibit a broad diversity of amino acid composition and

of tissue origin, their common characteristic is the high propensity to form water-insoluble β-pleated aggregates in mammals [14,15,16,17,18,19,20]. In addition to the fibrils, a constant constituent of amyloid deposits has been found to be normal serum glycoprotein. Polymerisation of relatively low molecular weight amyloid monomers leads to a highly ordered peptide folding process yielding high molecular weight aggregates that withstand proteolytic degradation. Potentially amyloidogenic proteins are produced in significant amounts by several organs [21,22,23]. Prevailing evidence indicates that amyloidogenic peptides and proteins exert a wide array of physiological functions under fine-tuned metabolic control [24,25,26,27,28]. Mutations and deleterious conditions, however, may dysregulate the synthesis and processing of amyloidogenic proteins, compromise their enzymatic degradation, and lead to pathological modification of peptide release [29]. Additionally, amino acid substitutions in and around the pre-mature amyloidogenic sequence may result in pathologically increased secretion of amyloid derivatives and may eventually be accompanied by abnormal folding of the proteins [30,31]. Taking these observations into account, it seems that pathological conditions boost abnormal amyloid-processing pathways and result in abundant generation of highly toxic amyloid derivatives which may be critical for the pathogenesis of a broad range of pathophysiologically distinct disorders with signs of peripheral and/or central nervous system dysfunction.

The main histological feature of AD is the presence of extracellular starch-like senile plaques [15,32,33,34,35] with different degrees of maturation [36]. Plaque formation persists over a long period of time and begins as amorphous, largely non-filamentous aggregates of Aβ. The initial form is called "diffuse plaque" which can be identified by sensitive silver staining methods (e.g., the Gallyas' silver stain). In the next step of maturation, diffuse plaques become increasingly fibrillar the plaque is called "neuritic plaque" or "senile plaque". It is a denser deposit of fibrillar amyloid with β-sheet structure, and shows thioflavin-S and Congo Red staining. Neuritic plaques are often surrounded by degenerating neurites [34,37]. Besides AD, formation of Aβ-containing senile plaques also occurs in Down's syndrome, during normal aging, and to a lesser extent after traumatic brain injury [38]. The extracellularly located plaques subsequently cause formation of intracellularly located neurofibrillary tangles, another essential feature of the AD brain [39]. Neuropathologically, AD is characterised by widespread neuronal death in brain regions subserving learning and memory formation, like the neocortex, amygdala, anterior thalamus, basal forebrain

cholinergic system and monoaminergic brain stem systems. A characteristic pathology occurs in hippocampus and medial temporal lobe. Pyramidal neurons are severely affected in AD [1,40].

Aβ is a proteolytic cleavage product of its precursor, termed amyloid precursor protein (APP) (Figure 1.). Aβ[1-42] has the following structure:

Asp-Ala-Glu-Ohe-Arg-His-Asp-Gly-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly$^{25}$-Ser-Asn-Lys-Gly-Ala-Ile$^{31}$-Ile-Gly-Leu-Met$^{35}$-Val-Gly-Gly-Val-Val-Ile-Ala

APP is a transmembrane protein [44] and is expressed as four major isoforms in humans, containing 695, 717,751 and 771 amino acid residues [41,42]. Generation of Aβ from the APP requires proteolytic cleavage by two proteases, β- and γ-secretase. APP is cleaved either by α-secretase resulting in $sAPP_{\alpha}$ (nonamyloidogenic pathway) , which might have a neuroprotective role, or by the protease BACE, identified as β-secretase, and then γ-secretase to yield Aβ [43]. Determination of the amino acid sequence of Aβ made possible the identification of APP gene which is located on chromosome 21 [44]. Aβ exists in two mature isoforms in the AD brain: the cerebral vasculature consists of 39-40 amino acid residues (Aβ[1-40]), senile plaque-forming Aβ comprises 42-43 amino acids (Aβ[1-42]) [15,45].

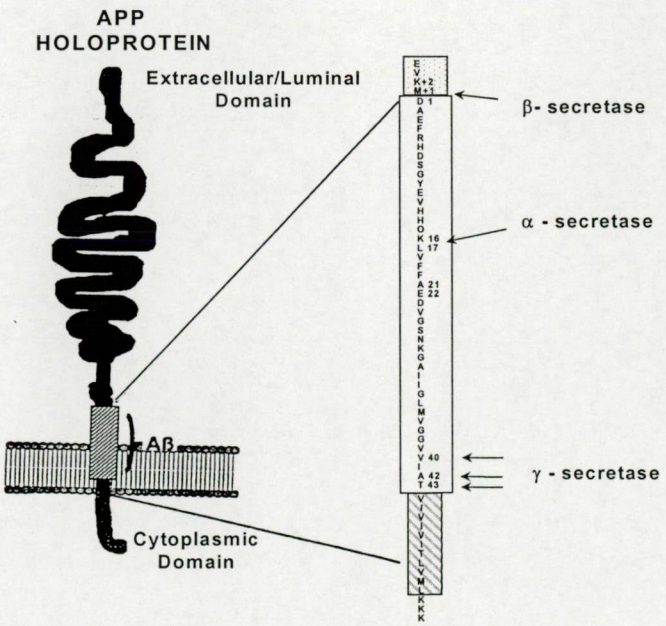


**Figure 1.** Structure and cleavage of APP molecule.

Diffuse Aβ deposits are thought to precede the formation of other neuropathological characteristics, involving neurofibrillary tangles [46] and abnormal phosphorylation of cytoskeletal proteins [47,48]. Aβ directly affects tau-phosphorylation, which may be a critical intracellular step of Aβ toxicity. The "tau and tangel hypothesis " argues that tangles are in fact central to AD. Tangles are comprised in of the microtubule associated protein tau, normally expressed in axons but which in AD becomes highly phosphorylated and aggregated into abnormal filaments in the cell body.

### 2.1.1. Role of APP in brain

APP represent a family of ubiquitous transmembrane glycoproteins, which span the lipid bilayer once and is expressed by activated cells that participate in cell-cell interaction. Neurons, the primarily affected cells in AD, are the only cells in brain that basically express APP. In neurons, newly synthesised APP is transported along the axon in a kinesin-I-dependent manner and subsequently and transcytosed to the somatodendritic compartment [49]. On the molecular level, APP has binding sites for cell surface receptors and for molecules of the extracellular matrix such as collagen, laminin, heparansulfate proteoglycans, and glycosaminoglycans (GAG) which suggests a general function for APP in regulation of cell and synaptic interactions [50]. APP was also found to bind zinc(II) and copper(II) at two distinct sites [51]. Some studies revealed that APP has an enzyme-like activity and reduces copper(II) to copper(I) while APP itself undergoes oxidative modification [52,53].

### 2.1.2. Role of aggregation

Above a threshold concentration, Aβ undergoes highly ordered protein aggregation in aqueous solutions resulting e.g. in the formation of senile plaque in AD [30]. Aggregated Aβ can be detected in low concentrations in the CSF and in the general circulation of AD patients, and may not only be the end-product of APP of neural origin but also of blood-born APP [7]. Aβ, released in a monomeric form in the extracellular space, begins a self-aggregation process, which, through the transient formation of several discrete intermediate Aβ species, leads to Aβ condensation as extremely insoluble protein fibrils with characteristic morphological features (80- to 150 Å fibrils) [30]. Shortly after its release to the extracellular

space in nM – pM concentrations, Aβ may exist as monomer. During the highly ordered process of Aβ fibrillogenesis, discrete but transient Aβ dimers, tetramers and intermediate oligomer species (termed protofibrils), exist between the two stable states of Aβ, the monomeric protein and the fibril. Intermediary protofibrils appear early during fibrillogenesis and disappear when fibrils appear [54,55,56,57,58,59]. Low molecular weight Aβ (monomers and dimers) do not alter the normal metabolic activity of neurons in culture [56]. Aβ[1-40] and Aβ[1-42] assemble through analogous steps, although Aβ[1-42] protofibrils are assembled more rapidly. The increased polymerization rate of Aβ[1-42] may be explained by increased hydrophobic interactions, such as by the formation of a longer β-sheet in the C-terminus, and by folding of this sheet over the assembly core [55]. Aggregated Aβ also can be detected in low concentration in the CSF and in the general circulation of AD patients [7,60]. *In vitro* aggregation of Aβ peptides results in the formation of identical, fibrils with those found in AD [61,62,63,64]. While Aβ[16-20] sequence seems to be essential to Aβ- Aβ binding [65], stabilization of the fibrillar state of Aβ is confined partly to a β-turn of Aβ[26-29] region and to hydrophobic interactions in the 29-42 domain [66,67]. Aggregation of the Aβ is strongly affected by concentration, pH, temperature, interactions of Aβ with essential membrane components, low concentrations of $Cu^{2+}$ and $Zn^{2+}$ions etc. [68,69]. Study of the influence of the different parameters on the aggregational properties of Aβ and their synthetic analogues can reveal essential data to the prevention of the undesirable transformations. Standardization of the aggregation grade of Aβ peptides is also essential for reproducible biological experiments.

### 2.1.3. Decline of the cholinergic system

Cholinergic neurons of the basal forebrain provide dense projections to the cerebral cortex and limbic system [70,71], and thereby modulate memory acquisition, storage and retrieval. One of the striking neuropathological markers of AD, is the widespread loss of cholinergic neurons of the medial septum and magnocellular nucleus basalis [5]. As the decline of cholinergic activity – decreased cholin-acetyltransferase (ChAT) activity and inhibition of the acetylcholine (ACh) release–, correlates with the progression of memory impairment in AD, the "cholinergic hypothesis of memory dysfunction" was postulated [72].

However, degeneration of cholinergic projection neurons is likely due to the loss of their synaptic contacts in post-synaptic target regions, like the hippocampus and neocortex, known to be primarily deranged in AD. Other neurotransmitter systems (e.g. serotoninergic, noradrenergic, glutamatergic) are also severely affected in AD.

### 2.1.4. Inflammation and free radicals

In the human brain several cell types are capable of initiating and amplifying a brain specific inflammatory response involving the synthesis of cytokines, prostaglandins and oxygen free radicals. In AD, signs of an inflammatory activation of microglia and astroglia are present inside and outside amyloid deposits [73]. Cell culture and animal models suggest an interactive relationship between inflammatory activation, reduced neuronal functioning and deposition of amyloid [74].

Damage to eventually all biomolecules by reactive radicals, particularly by oxygen and nitrogen species, is referred to as oxidative stress. The radical-mediated breakdown of membrane components is termed lipid peroxidation. [75]. Aging is associated with the temporal enhancement of oxidative damage to neurons, and accelerated oxidative neuronal damage is evident in AD, an oxidative stress hypothesis was proposed to describe cellular events leading to neuronal loss in AD [76,77]. Aβ induces oxidative stress to neurons by acting as a free radical following distortions of its electronic structure, by interacting with membranous cellular components by its lipophylic nature, and by initiating receptor-mediated pro-inflammatory signaling pathways. Aβ may generate free radicals in aqueous solution [78]. The presence of Met residue at position 35 of Aβ was regarded as pivotal: substitution or lack of Met-35 significantly abrogates Aβ toxicity, free radical formation and protein oxidation [79,80].

### 2.1.5. Receptor-binding theory

Exposure of nerve, glial and endothelial cells to Aβ causes modification of a wide variety of cellular functions, which differ depending on the stereochemical characteristics, aggregation state, and concentration of the Aβ fragment applied. While at nM – pM range Aβ concentration promote neuronal differentiation and increase neuronal viability [28,81,82], Aβ

is toxic to neurons at μM range and induce necrotic [83] or apoptotic cell death [84,85] in a dose-dependent fashion. For low concentrations of Aβ, a direct neuromodulatory role affecting cholinergic neurotransmission in the brain was proposed [86,87]. *In vivo* investigations indicated neurotoxic properties of μM Aβ concentrations on cholinergic neurons of the basal forebrain [88,89]. It was assumed that Aβ exerts its pathologic effects through cell-surface receptors. Since Aβ destabilises the intracellular $Ca^{2+}$ homeostasis [90,91], and augments the generation of reactive oxygen species (ROS) [92,93], a number of receptors were proposed as molecular targets for Aβ. Exposure of nerve cells to Aβ activates $Ca^{2+}$ permeable receptor channels which results an increase of intracellular $Ca^{2+}$ concentration [29]. Excessive activation of NMDA subclass of glutamate receptors was reported to lead to excitotoxic neuronal death under pathological conditions [94,95]. Besides neuronal receptors, endothelial and glial receptor subclasses were also recognized as pivotal for the uptake and transcytosis of Aβ at the BBB from the general circulation [96] and for the activation of inflammatory mechanisms in the brain [97]. In this regard, the receptor for advanced glycation endproducts (RAGE), which is a member of the immunoglobulin superfamily of cell-surface molecules, and the type A macrophage scavenger receptor (MSR/A) received much attention [98,99,100].

### 2.1.6. The aluminium theory

One decade ago, it was hypothesized that aluminum plays a role in the etiology of Alzheimer's disease. Because this theory could not be proved, the scientific opinion refused it by now. There are results suggesting that the total amount of exposure and lifetime occupational exposure to aluminium are not likely to be important risk factors for Alzheimer's disease [101]. None of the extensive European and American case-control studies have found a relationship between AD and aluminum antacids intake.

### 2.1.7. Risk factors

The epidemiological study of AD with special emphasis on its genetic causes provided us with important findings and a very detailed knowledge of the risk factors of the disease in the past few years. The following risk factors were found:

**a. Age** is the most important risk factor for dementias of all kinds. The prevalence of AD shows an exponential rise with age. The number of cases double in every 5 years in the population aged over 65 years [102].

**b. Genetic causes** constitute the second most important risk factor for the disease [102]. Different genetic factors contribute to AD, some of them being directly and invariantly causative of the disease, producing familiar AD (FAD).

Clinically typical AD can cluster in families and can specifically be inherited in an autosomal dominant fashion. Determining how frequently genetic factors underlie the disease is difficult in a late-onset disorder such as AD. Moreover, the clinical manifestations are generally quite similar or almost indistinguishable from those of the sporadic cases, although some families may show distinctive clinical signs [103]. The recognition that polymorphic alleles of apolipoprotein E (apoE) can predispose strongly to the development of AD in the 60s and 70s suggests that other polymorphic genes could predispose to the disorder but would be difficult to detect in genetic epidemiological studies, because they do not always produce the disease and will thus not show high penetrance [104,105,106,107].

The first specific genetic cause of AD to be identified was the occurrence of missense mutations in APP [108]. Despite extensive genetic surveying, such mutations have only been confirmed in some two dozen or so families world-wide. Now, 4 well established genes are associated with AD (Table 1.).

| Chromosome | Gene Defect | Phenotype |
|---|---|---|
| 21 | β-APP mutations | ↑ Production of all Aβ peptides or Aβ[1-40] peptides |
| 19 | ApoE4 polymorphism | ↑ Density of Aβ plaques and vascular deposits |
| 14 | Presenilin 1 mutations | ↑ Production of Aβ[1-42] peptides |
| 1 | Presenilin 2 mutations | ↑ Production of Aβ[1-42] peptides |

**Table 1.** Genetic factors predisposing to Alzheimer's disease: relationships to the Aβ phenotype.

**c. Precipitating factors**: the existence of certain situations is often found in the prehistory of AD patients. The most important ones are the following factors:

- lack of education [102,109];

- head injury (defined as one sufficient to produce unconsciousness) up to 30 years prior to the onset of AD, either accidental or repeated [110,111];

- some illnesses, including depression (occurring at 10 or less years before AD onset), atherosclerosis, coronary disease, diabetes and hypothyroidism, were also shown to increase the prevalence of AD.

### 2.1.8. Diagnostics for AD

AD described clinically as a progressive, degenerative and irreversible process, is only one of the diseases that cause dementia. Therefore diagnostics would be important to rule out treatable diseases other then AD (e.g. hypothyreosis, depression, psychosis, head trauma, neurosyphilis, tumours, B12 vitamin deficiency, etc.) and to enable the initiation of an adequate therapy. Although numerous clinical signs have been described, there are no satisfactory, sensitive, and specific clinical tests for AD so far, unambiguous evidence can provide only the histopathological autopsy. Diagnostics may become even more important in the future as subtypes of AD may be teased out that respond better to particular therapeutic strategies.

The Alzheimer's Disease Society described in 1998 seven stages in progress of AD: in the first stage, there is no apparent cognitive decline, the third stage is described as a state with mild cognitive decline, getting lost, low performance, concentration deficit, and the seventh stage characterised by late dementia.

Clinical attempts focus especially on the possible earliest diagnosis. For example there are studies that suggest in patients with mild cognitive impairment, olfactory identification deficits, particularly with lack of awareness of olfactory deficits, may have clinical utility as an early diagnostic marker for AD [112].

### 2.1.9. Prevention and therapy attempts

There are only few treatments available to improve cognitive and behavioural symptoms, as well as therapies that may slow the progression of the disease somewhat (cholinesterase inhibitors, nicotinic and muscarinic acetylcholine receptor ligands, and other cognitive enhancers), but there is no cure for AD. Soon after the definition of numerous fundamental factors that may contribute to the development and progression of AD, including – without attempt of full coverage - genetic factors [113], apoE polymorphism [114],

cholinergic decline [115], τ hyperphosphorylation [116], Aβ toxicity, Aβ aggregation and β-sheet breaker (BSB) peptides, the search for potential neuroprotective intervention yielded several distinct directions [117,118].

Epidemiological studies have documented a reduced prevalence of AD among users of some nonsteroidal anti-inflammatory drugs (NSAIDs) [119,120], (such as ibuprofen, indomethacin, and sulinac sulfin). This effect seems not to be mediated by inhibition of cyclooxygenase activity [121]. Aspirin (the best known NSAID) had an unconvincing effect in epidemiological studies. Similarly, other classes of anti-inflammatory compound (e.g. hydroxy-chloroquine or prednisone) did not show benefits to AD patients [122]. Vitamin E as a lipid-soluble antioxidant has been reported to slow functional loss in AD patients. Vitamin C, another antioxidant, might be a potential inhibitor of neuritic plaque formation 123].

Application of $Ca^{2+}$ antagonists is favoured on the recognition that Aβ initiates a rapid elevation of $[Ca^{2+}]_i$ [124,125]. Several studies indicate that women who take oestrogen after menopause have an unexpectedly low incidence in AD [126].

One potential route for therapy involves the immunization of patients against the Aβ. Resulting from earlier research on transgenic mice [127], Elan Pharmaceuticals began clinical trials of a vaccine, AN–1792. But some scientists warn that AN-1792 could cause a dangerous autoimmune response in humans [128].

The possibility of neuroreplacement therapies in case of neurodegenerative disorders (such as AD and Parkinson's) is supported by the existence of neuronal stem cells in the adult human brain [129].

## 2.2. Stability studies of Aβ peptides

Stability studies are very important in drug research: as more peptides and proteins are targeted to be used as drugs, their propensity toward degradation should be examined and appropriate stabilising modifications made as required. "Aging" of Aβ peptides is a common method before *in vivo* experiments [130,131,132]; this is a long (1-3 days) aggregation process at pH=7.4 for fibril formation. *In vitro* investigations of Aβ neurotoxicity in cell and tissue cultures proceed for several hours or even days [133, 134] at pH=7.4, 37 °C.

Deamidation of asparagine (Asn) residues, to yield β- (iso-aspartic) or α- aspartic acid (Asp) residues in place of the original Asn, is a major nonenzymatic degradation pathway for many proteins [135,136]. This intrinsic instability of Asn is proposed to serve as a "molecular clock". Oxidation of metionin (Met) also could occur.

# 3. Materials and methods

## 3.1 Synthesis and purification of peptides

All peptides were synthesised by solid phase technique, mostly manually, but in some cases on ABI 430A (Applied Biosystems Co.) automated peptide synthesiser, using Boc- or Fmoc-chemistry. We applied a new version of the standard Fmoc-chemistry, therefore this new method will be more detailed described in the Results. Most peptides were synthesised with amidated C-terminus, but some of them were prepared with free carboxyl C-terminus, depending on the linker of the used resin. Protected amino acids and resins were purchased from Bachem and used without further purification. Protected amino acids used in the syntheses were of the L-configuration. Coupling agents were from Richelieu Biotechnologies (HOBt) or from Fluka (DCC). TFA was from Fisher Scientific, and the remaining reagents from Aldrich. Solvents (DCM, DMF, $CH_3OH$ and $CH_3CN$) were from Merck.

### 3.1.1. Peptide synthesis by Boc chemistry

The syntheses involving Boc-chemistry of peptides with amidated C-terminus were carried out on MBHA resin. Peptides with free carboxyl group at the C-terminus were synthesized on Merrifield resin. The first amino acid was coupled to the Merrifield resin using an oil bath at 70 °C for 48 hours. The following side-chain protections were used: Arg(Tos), Asp(OcHex), Glu(OcHex), His(Z), Lys(2ClZ), Ser(Bzl), Tyr(2BrZ); the side chains of Asn and Gln were unprotected.

In case of manual synthesis, the coupling reactions were achieved with a 3-fold excess of Boc-amino acids with DCC (3 equiv.) as activating agent in DCM or DCM – DMF (1:1) or

performed HOBt ester for Asn and Gln in order to avoid side-reactions. After a coupling time of 2 hours, the completeness of acylation was monitored at each stage by Kaiser's standard ninhydrin test [137]. The test reaction is quick and easy to carry out, but some deprotected amino acids (Asn, Asp, Ser and Pro) do not show the typical dark blue colour. For monitoring the coupling and deprotection of Pro residues, the choranil test was used [138]. In cases where incomplete coupling was found, the coupling procedure was repeated or acetylation was carried out before the removal of the $N^{\alpha}$-amino protecting group prior to the coupling of the next amino acid. Acetylation was performed with 30% acetic anhydride in DCM for 10 and 20 minutes. Intermediate Boc removal was achieved with 50% TFA in DCM (containing 0.5% dithiothreitol, if Met was present in the peptide sequence) followed by neutralisation with 10% TEA in DCM. After completion of the synthesis and removal of the $N_{\alpha}$-Boc protecting group from amino-terminus, some peptides were acylated with propionic acid or with succinic acid using symmetrical anhydride method. Final deprotection as well as the cleavage of the peptides from the resin were performed with anhydrous hydrogen fluoride in the presence of 8% anisol, 2% dimethylsulfide, 2% p-cresol and 2% thiocresol as scavengers at 0 °C for 60 min. For 1.0 g peptide-resin 10 ml HF was used. After the removal of the hydrogen fluoride under a stream of nitrogen and in vacuum, the free peptides were precipitated with diethyl ether, filtrated, washed with diethyl ether and extracted with 50% aqueous acetic acid (short peptides) or with 95% TFA (long peptides), diluted with water, and lyophilised.

### 3.1.2. Purification of peptides

After lyophilization, about 150 mg crude peptide was purified by using a Shimadzu LC-8A preparative HPLC system. A PrePak 300x47 mm VYDAC column packed with C18 silica gel (300 Å pore size, 15-20 μm particle size) for short peptides and a Backbond WP C4 column (300x47 mm, 300 Å pore size, 15-20 μm particle size) for long peptides. The column was eluted usually with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 80% aqueous ACN in a linear gradient mode. For semipreparative scale purification a Knauer HPLC apparatus (Knauer GmbH.) with a variable wavelength UV detector was used, equipped with a Nucleosil 300 $C_4$ (16x280 mm, 300 Å pore size, 10-20 μm particle size) for long peptides or with a BST SI-100S $10C_{18}$ semipreparative column for short peptides. For

FT-IR spectroscopy, peptides were purified with a solvent system consisting of (A) 0.05 M $NH_4OAc$ and (C) 0.05 M $NH_4OAc$ in 60% ACN. Extinction was monitored usually at $\lambda = 220$ nm. The fractions were checked by analytical HPLC and those with purity higher than 95% were pooled and lyophilised and stored at –20 °C.

## 3.2. Analytical characterisation of peptides

### 3.2.1. Analytical HPLC

A Knauer HPLC apparatus was used for analytical HPLC control of the peptides equipped with a Lichrosorb 5RP-$C_{18}$ (250 mm x 4 mm) reversed-phase analytical column or with a Nucleosil-300 $C_4$ (250 mm x 4 mm) reversed-phase analytical column for peptides containing more than 20 amino-acids.

### 3.2.2. Amino acid analysis

About 500 μg samples of purified peptides (with purity more than 95%) in sealed glass tubes and vacuum were hydrolysed in 6N HCl for 24 h at 110 °C. The analysis were done on a Hewlett-Packard Amino Quant automatic amino acid analyser, equipped with an Amino Quant $C_{18}$ (200 mm x 2.1 mm) hipersyl column, using OPA (ortho-phthalo-dialdehyde) derivatization.

### 3.2.3 Mass spectrometry (MS)

Electrospray ionisation (ESI) mass spectra were recorded on a FinniganMat TSQ 7000 mass spectrometer. Fast atom bombardment (FAB) mass spectra were obtained on a modified FinniganMat 212/AMD 5000 double-focusing mass spectrometer and a VG-ZAB 2SEQ hybrid tandem mass spectrometer, both equipped with a liquid secondary ion mass spectrometer source ($Cs^+$ ion gun).

3.2.4 Liquid chromatography combined with mass spectrometry (LC-MS)

For stability investigations, LC-MS measurements were carried out in order to identify all peptide fragments formed during the incubations in vials. The contents of vials were lyophilised for a tender concentration. The HPLC apparatus was equipped with a Nucleosil 300 Å $5C_{18}$, 2x100 mm analytical column, gradient 0→60% B over 30 min, eluted with A = 0.1% TFA and B = 70% ACN + 0.1% TFA at flow rate of 0.2 ml/min. Extinction was monitored at $\lambda$ = 220 nm. The outlet of HPLC apparatus was connected to the inlet of the ESI-MS apparatus (FinniganMat TSQ 7000 mass spectrometer). MS scans in the necessary $M/z^+$ ranges (typically 150 to 2000) were performed every 2 seconds.

**3.3. Secondary structure investigation of βA peptides:** FT-IR spectroscopy

FT-IR spectra were performed on a Nicolet Impact 410 spectrometer. The peptides were dissolved in HFIP (hexafluoro-2-propanol) or in DMSO and then diluted with phosphate buffered saline.

**3.4. Stability investigations of Aβ[25-35]**

3.4.1. Incubation of Aβ[25-35]

The stability investigation of Aβ[25-35] peptide was carried out at 37°C in double-distilled, ion-exemptioned water, under physiological conditions at pH = 7.4 and in pH = 8.0 phosphate buffer made of double-distilled, ion-exemptioned water in the presence and without $CaCl_2$. In both cases the concentration of Aβ[25-35] (with purity higher then 99%) was 0.1 mM. The composition of the physiological solution was: NaCl 144.0 mM, KCl 4.0 mM, $CaCl_2$ 1.8 mM, $NaH_2PO_4$ 0.33 mM, $MgCl_2$ 0.53 mM and HEPES buffer (active in the pH range 6.0-8.5) 5.0 mM. In order to prevent biological degradation, 0.02 % of $NaN_3$ was used in the experiments. The solutions were pipetted into sterilised vials with single-used sterile

syringes; then the vials were sealed. Samples were taken in every hour for one day and then daily.

3.4.2. Product analysis

The HPLC-analysis of the samples was carried out by using an analytical Knauer HPLC system equipped with a Lichrosorb 5RP-18 (250 mm x 4 mm) reversed-phase column. The column was eluted with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 80% aqueous ACN in linear gradient mode (0 - 60% B in 30 minutes, flow 1.0 ml/min). The eluent was monitored at 220 nm. The different peaks were collected into vials, lyophilised and identified by electrospray mass spectrometry (ES-MS, FinniganMat TSQ 7000 mass spectrometer).

## 3.5. Biological investigations of Aβ peptides

3.5.1. Cell culture, treatment, and fluorescence measurements

Primary cultures of rat astrocytes were prepared from the cerebral hemispheres of new-born rats by mechanical dissociation. The cells were plated onto glass coverslips pre-treated with poly-L-ornithin and fibronectin, and were placed on 12-well culture dishes and cultured for 4-6 weeks at 37 °C in humidified 5% $CO_2$ atmosphere. At this stage, about 95% of the cells were positive by immuno-cytochemical staining. The cells on coverslips were exposed to 1 μmol/l final concentration of the different βA peptides for 8 hours. Intracellular loading of Fura-2-AM was attained by incubating the cells with 2 μM Fura-2-AM during the final 30 min of the 8-hour, 37 °C incubation period. The cells on coverslips were then washed and the coverslips were transferred to cuvettes containing Hepes Buffer Tyrode at 25 °C for measurements. Fluorescence measurements in standard 1x1 cm cross section quartz cuvettes were performed with a Hitachi F-2000 spectro-fluorimeter. Ratiometric fluorescence determinations were utilised excitation at 340 and 380 nm, using emission wavelength of 495 nm at 10 nm bandwidths and the 367 nm-excitation was taken as a $Ca^{2+}$-insensitive wavelength [139].

3.5.2. Investigations of peptides by microdialysis *in vivo*

For experiments young adult male Whistar rats were used: a concentric microdialysis probe was implanted into the right MBN at standard co-ordinates. Inlet and outlet tubes of the probe were run through a liquid swivel to allow collecting dialysis samples from freely moving conscious animals. Dialysis was started 24 h after probe implantation. Artificial cerebrospinal fluid (ACSF) served as vehicle throughout the experiments. Both Aβ[1-42] and the peptide with code ZM6 were used in equimolar concentration (200 μM) and solubilized in ACSF to which 0.1% TFA had been added; vehicle + TFA-dialysed animals served as controls. After an equilibration period, ZM6 was infused for 60 min, which was followed by a 40 min infusion of Aβ[1-42]. Outflow fractions were collected to determine the excitotoxic action of Aβ[1-42] and its prevention by equimolar ZM6. Extracellular amino acid levels were determined by HPLC analysis.

3.5.3. Behavioural tests

Novelty-induced spontaneous activities of the animals were assessed 24 h post-surgery [140]. Each test lasted for 3 min, during which latency to the start of exploration, the degree of horizontal ambulation, and the number and total time a rat spent in an upright position were recorded. Animals were also tested for discriminative learning in a one-way step through passive avoidance task 14 days post-surgery. In the training trial (day 12) the animals were placed in an illuminated chamber to explore the boxes. The latency to step into the dark compartment was recorded. On the second day of the test (day 13) a mild foot shock (1mA, 3 s) was delivered through the grid floor upon entry into the dark compartment. Retesting was performed 24 h later(day 14) and the latency to step into the dark chamber was recorded (post-shock latency) within a total of 3 min retesting period.

3.5.4. Measurement of membrane potential changes by flowcytometry

Immortalized striatal cell cultures of new-born rats (cell line M26-1F) were prepared and suspended [141]. 400 μl cell suspension was incubated with 4 μl of peptides ($10^{-4}$ M) for 10 min, then 4 μl bis-oxonol was given. The membrane potential of the mixture was measured

by flowcytometer after 2 min. Each experiment started and finished with control measurements. The measurements were performed at room temperature (22 ± 2 °C) on a Coulter EPICS 750 flow sorter supplied with a 14 mW Ar laser (488 nm). The gate number was $10^4$ cells. Histograms obtained by representation of cell number vs. fluorescens intensities and dot – plot figures were compared and analysed.

3.5.5. Measurement of the neurotoxic effect of amyloids and the neuroprotective effect of Aβ-antagonists with MTT test on SH-SY5Y neuroblastoma cells

Aβ peptides were dissolved in a medium which did not contain Phenyl-red. These stock solutions were sonicated for 10 minutes, then aggregated for 4-5 days at 4 °C (10 minutes daily sonication) then the stock solution was homogenized. The neuroblastoma cells were mechanically agitated in a Phenyl-red free medium then collected in a dish. The cells were counted (30000 cells/100 µl), pipetted into sterile Eppendorf vials, then incubated for 3h at 37 °C in humidified 5% $CO_2$ atmosphere. The cells were treated with the Aβ peptide containing stock solutions (warmed up to 37 °C). The treated cell-suspension were pipetted into plates containing 96 wells (100 µl/well) and incubated for another 24h at 37 °C in humidified 5% $CO_2$ atmosphere. Each well was supplemented with 10 µl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) stock solution (4 mg/ml PBS). The plates were incubated again for another 3h at 37 °C in humidified 5% $CO_2$ atmosphere. The cell culture medium was carefully removed from the cells. The MTT treated cells were homogenized and then the MTT-formasan was diluted with a 4:1 ratio mixture of DMSO/EtOH (100 µl/well). The plates were again sonicated for 10 min. After this procedure the cells were measured with an Elisa Reader photometer at 550/620 nm. The results were statistically analyzed.

# 4. Results

## 4.1. Synthesis and purification of peptides

| | |
|---|---|
| $A\beta_{1-40}$ | DAEFRHDGGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-$NH_2$ |
| $A\beta_{1-42}$ | DAEFRHDGGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVAI-$NH_2$ |
| MOD3 | DGEGRHDSGGEEHHQKEGEGGEDEGSNKGAIIGLMEGGEGEG-$NH_2$ |
| MOD4 | DAEFRHDGGYEVHHQKLVFFAEDVGSNKGAAAGAAVGGVVIA-$NH_2$ |
| $A\beta_{25-35}$ | GSNKGAIIGLM-$NH_2$ |
| $A\beta_{27-35}$ | NKGAIIGLM-$NH_2$ |
| $A\beta_{28-35}$ | KGAIIGLM-$NH_2$ |
| $A\beta_{29-35}$ | GAIIGLM- $NH_2$ |
| $A\beta_{31-35}$ | IIGLM-$NH_2$ |
| $A\beta_{31-35}$-OH | IIGLM-OH |
| $A\beta_{25-30}$ | GSNKGA-$NH_2$ |
| $A\beta_{25-30}$-OH | GSNKGA-OH |
| $A\beta_{24-28}$ | Suc-EGSNK-$NH_2$ |
| ZM6 | Pr-IIGL-$NH_2$ |
| R-ZM6 | RIIGL- $NH_2$ |
| Aβ-Tjernberg | KLVFF-$NH_2$ |
| KLV6 | KLVGF- $NH_2$ |

**Table 2.** Sequences of the synthesized Aβ, APP and modified Aβ peptides.

### 4.1.1. Synthesis of Aβ[1-40] peptide with Boc-chemistry

The synthesis was carried out on an MBHA resin with 0.40 mmol/g substitution. After the cleavage of the peptide from the resin with HF, the purification was performed in several steps. Poor solubility of the crude peptide and the high tendency of the peptide solution to form colloidal or even visible precipitates caused difficulties during the post-cleavage work-up. To minimise losses, lyophilization and a new dissolution before the first purification was avoided. After the post-cleavage work-up, the peptide solution was immediately purified by HPLC. To apply the peptides on HPLC column, they were dissolved in 60% ACN in distilled water containing 0.1% TFA at 3 mg/ml concentration using sonication. The solution was quickly filtered from the resin and diluted threefold with water, filtered through a bed of Hyflo, and injected on a Backbond WP $C_4$ cartridge for a first preparative purification. The

used gradient was 25% →35% B in 10 min and then 35% → 45% B in 30 min. The average purity was 60 %. After that 91.5 mg of a peptide fraction with 70% purity was purified with semipreparative HPLC, 27.0 mg peptide with 90% purity was obtained. Finally, this 27.0 mg peptide was purified as well and gave 6.2 mg peptide with 98% purity. According to necessity, additional lyophilised peptide fractions were purified. In the absence of the $C_4$ semipreparative column the purification was repeated on a BST SI-100S $10C_{18}$ semipreparative column with a gradient of 30% → 40% B in 10 min and then 40% → 70% B in 60 min.

4.1.2. Synthesis of Aβ[1-42] peptide with Fmoc-chemistry

We have found that the main problem of the solid-phase Aβ-synthesis is the aggregation of the growing peptide chains within the polystyrene beads, which cause enormous coupling problems during the synthesis. Coupling should be repeated sometimes 3- or 4-times for getting a good acylation grade. As a consequence, we have worked out a new, optimized method for the synthesis of long Aβ peptides.

As an example, Aβ[1-42] was synthesized on trimethoxy-benzhydrylamine resin (Rink resin) using standard Fmoc-chemistry ($N^{\alpha}$-amino protection: Fmoc; side chain protections: $Bu^t$ (Thr, Ser, Tyr), Boc (Lys), Pbf (Arg), Trt (His), $OBu^t$ (Asp, Glu). The only but very important change was the use of 10% anisol in all the solvents during all the couplings. Anisol (similar to DMSO) can deaggregate the peptide chains also within the polymer bead. Using anisol, couplings had not be repeated in most cases, except the middle sequence of the molecule, there couplings were repeated. The Aβ[1-42] was cleaved from the resin with 50% TFA in DCM, using 2% $H_2O$ and 10% anisol as additives. After cleavage, the peptide solution was evaporated in vacuum, then the peptide was precipitated with ether, filtered and redissolved in trifluoroethanol (TFE) : water = 2:1 and lyophilized. The crude product contained some impurities and was purified on a preparative C-4 Vydac column, using ACN gradient 25% → 35% in 30min. This method gave a very good yield (~15%) and a very pure product.

### 4.1.3. Synthesis of peptides *MOD3* and *MOD4*

*MOD3* and *MOD4* peptides were designed and synthesized as synthetic analogues of the Aβ[1-42] and then followed and compared their aggregation to the aggregation of Aβ[1-42]. The *MOD3* analogue was designed and synthesised in such sequence, which contains the toxic pentapeptide fragment Aβ[31-35] of the Aβ[1-42] peptide and the others are mainly hydrophilic residues. The *MOD4* peptide has the same amino acid sequence as Aβ[1-42] with the exception of the Aβ[31-35] fragment. The amino acids of the (31-35) fragment had been replaced by Ala with the exception of the Gly. Both syntheses were carried out manually using Boc-chemistry. First, the crude peptides were purified on preparative HPLC, equipped with a Vydac $C_{18}$ cartridge. The purified peptide fraction with 90% purity was submitted for further purification with a solvent system containing 0.05 M $NH_4OAc$ instead of 0.1% TFA, resulting in a product as an acetic acid salt. The purification was carried out on a Knauer HPLC apparatus, equipped with a BST SI-100S $10C_{18}$ and Knauer Lichrosorb RP18 semipreparative columns, respectively. The used gradient was 1% → 20% B in 10 min, and then 20% → 50% B in 70 min.

### 4.1.4. Design and synthesis of Aβ fragments: β-sheet breakers (BSBs) and functional antagonists

Aβ[25-35] shows the whole neurotoxic effect of Aβ[1-40] and Aβ[1-42] if aggregated [82]. On the other hand, the Aβ[16-20 ] sequence seems to be very important for aggregation ('seeding sequence") and the original sequence KLVFF ("Tjernberg peptide") can deaggregate Aβ peptides or prevent aggregation ("β-sheet breaker", BSB) [65,142].

Some years ago we have found a short Aβ fragment (31-34) which show a very low neurotoxicity, however, in all the *in vitro* and *in vivo* tests can prevent the neurotoxic effect of Aβ[1-42]. This peptide was called as functional antagonist – inverse agonist. The propionyl derivative of this short peptide proved to have a better neuroprotective effect (Pr-IIGL; ZM6) [143]. Using these peptide sequences KLVFF and Pr-IIGL as lead compounds, we have designed and synthesized short pentapeptides in order to have better BSBs and/or better antagonists.

The results were very interesting. If we change only one Phe to Gly in Tjernberg peptide, the resulting analog KLVGF shows only a very small BSB activity, however, it proved to be one of the best functional antagonist ever synthesized. In MTT-test (*in vitro*, SH-SY5Y neuroblastoma cell line) this peptide can protect neurons from the neurotoxic effect of Aβ[1-42] also in 1:1 molecular ratio (Figure 2.)! This KLVGF peptide serves now as a lead compound for further design of Aβ antagonists, peptides and peptidomimetics, and this work is the subject of a patent.

On the line we found an arginine analog of ZM6, RIIGL, which proved to be much more active antagonist then the mother compound (Figure 2.). The sequence RIIGL serves as lead for further design of antagonists (peptides and peptidomimetics) and this work is also subject of a patent.

Summarizing this work, we have found two pentapeptides, which show excellent neuroprotective effect as Aβ-antagonists.

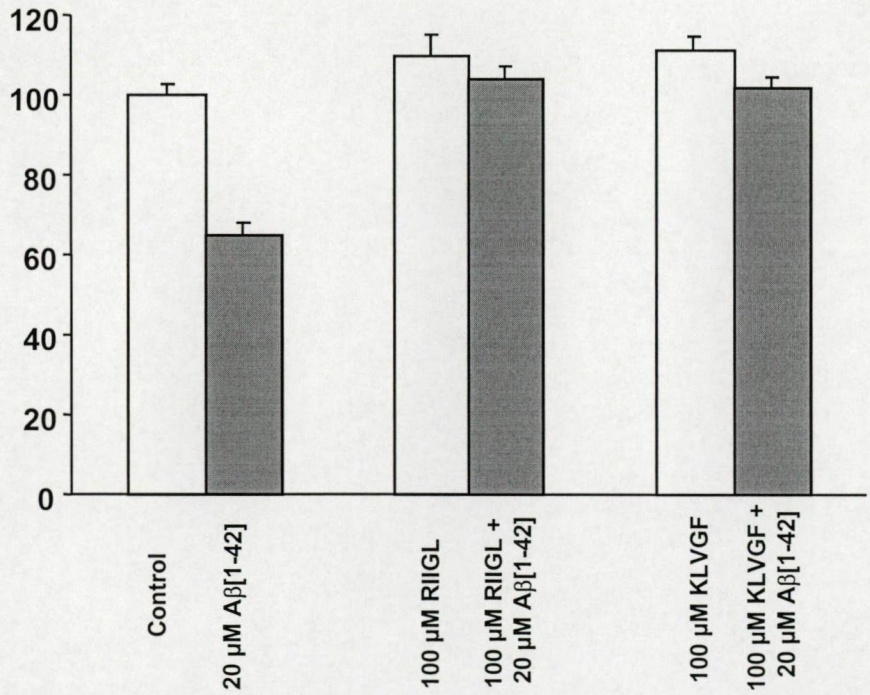


**Figure 2.** Optical density measurement of formasan for toxicity investigations of Aβ[1-42] and the protective effect of Aβ-antagonists.

## 4.2. Analytical characterisation of peptides

### 4.2.1. FT-IR spectroscopy

The aggregation of Aβ peptides were studied under different conditions (pH, temperature, concentration, etc.) in order to determine the effects of the conditions that were varied in the biological experiments. Since amyloid peptides exist in different conformation and aggregation states depending on the circumstances of preparation and purification, a standardisation method was applied. Peptides were dissolved in DMSO (which is widely used in biological experiments) or HFIP. Such stock solutions can be chosen as a unique reference start-point for aggregation and conformational studies, as amyloid peptides have been found to be monomeric, with a solvated and unordered (in DMSO), or α-helical (in HFIP) structure [144]. Dilution with PBSA at the required pH was used instead of lyophilization and redissolution because of the uncertainties in the lyophilization parameters. As time passed, upon the addition of PBSA (pD 7.4) to the DMSO solution of Aβ[1-40], a conformational transition began immediately, resulting in β-sheets, which became more associated and the interactions between them grows stronger. Aβ[1-42] exhibits properties similar to those of βA[1-40], but with higher amount of associated β-sheets. The 2D FT-IR studies are in good agreement with the finding that Aβ[1-40] displays less tendency to aggregate than Aβ[1-42], demonstrating the importance of the C-terminal region in the aggregation. The within 1 h random-to-sheet transformation was also demonstrated. 2D FT-IR analysis in the 24 h domain shows simultaneous helix-to-sheet and sheet-to-sheet transformation. From this it was deduced that the conversion of helical segments to β-sheets is slower than in case of the random structures. The intramolecular H-bonds of the α-helices may explain this finding.. Studies with samples heated at 90°C indicate the formation of β-sheets with strong H-bonds between the molecules. A highly aggregated state of the β-sheets was reached within 15 min, after which no change was detected. Samples incubated at 37°C after the heating experiment showed the same random-to sheet process as samples that had not been heated. These observations led to the conclusions, that Aβ[1-40] forms β-sheets which are stable even at high temperature, and these very stable sheets are formed from intermediate β-sheets. However, formation of these intermediate β-sheets is not favoured at 90°C.

The 2D FT-IR study demonstrated that the *MOD-3* peptide is not able to aggregate at all or it aggregates extremely slowly. The aggregation ability of the *MOD-4* and the Aβ[1-42] peptides are similar, however *MOD-4* shows a small amount of antiparallel β-sheet formation.

## 4.3. Biological investigation of Aβ peptides

### 4.3.1. Intracellular long-term elevation of $Ca^{2+}$ in rat astrocytes

The effect of different Aβ peptides on the intracellular $Ca^{2+}$ level was monitored by comparative fluorimetric studies of rat astroglial cells grown on coverslips. The cells were exposed to 1 μM concentration of the peptides for 8 h. After that, cells were found to be labelled satisfactorily with 2 μM Fura-2-AM for 30 minutes at 37°C by passive diffusion. Values of 340/380 fluorescence excitation ratio at 495 nm emission wavelength were measured. The steady-state fluorescence intensities using the $Ca^{2+}$-insensitive excitation wavelength of 367 nm, are presented in Table 3.

| Treatment | 340/380 fluorescence excitation ratio | Fluorescence excited at 367 nm | Number of coverslips |
|---|---|---|---|
| Control | 1.25 ± 0.06 | 355 ± 20 | 51 |
| Aβ[1-42] | 1.39 ± 0.04 | 367 ± 22 | 18 |
| Aβ[1-40] | 1.37 ± 0.06 | 360 ± 18 | 9 |
| Aβ[25-35] | 1.38 ± 0.05 | 358 ± 16 | 12 |
| Aβ[31-35] | 1.41 ± 0.04 | 373 ± 17 | 12 |

**Table 3.** Results of fluorescence measurements.

The ratio of the 495 nm fluorescence intensities excited at 340 nm and 380 nm (340/380 fluorescence excitation ratio) was consistently higher in treated cells as compared to their untreated counterparts. Importantly, no significant alteration was detectable in Fura-2

fluorescence using the $Ca^{2+}$-insensititive excitation wavelength of 367 nm, indicating that the observed response reflects a real change in the $Ca^{2+}$ concentration in the cells. During the 8-hour incubation period, the treated and untreated cell populations were cultured in media of identical composition except for the presence or absence of the β-amyloid peptides. The observed difference in the 340/380 fluorescence excitation ratio of Fura-2-loaded cells can be exclusively associated with the presence or absence of Aβ peptide. All the peptides investigated induced similar cellular responses, suggesting that the [31-35] region of the peptide sequence could be the putative active centre of the molecule.

Co-treatment of the cells with equimolar concentration of ZM6 peptide, an analogue of the [31-34] sequence, was found to antagonise the Aβ[1-42]-induced increase in the 340/380 fluorescence excitation ratio, leaving the $Ca^{2+}$-insensitive 367 nm fluorescence unchanged (Table 4).

| Treatment | 340/380 fluorescence excitation ratio | Fluorescence excited at 367 nm | Number of coverslips |
|---|---|---|---|
| Control | 1.22 ± 0.06 | 355 ± 18 | 16 |
| Aβ[1-42] | 1.41 ± 0.05 | 359 ± 21 | 16 |
| ZM6 | 1.26 ± 0.04 | 361 ± 17 | 16 |
| Aβ[1-42] + ZM6 | 1.28 ± 0.04 | 358 ± 19 | 16 |

**Table 4.** Effect of ZM6 peptide on the Aβ[1-42] induced change in the 340 nm/380 nm fluorescence excitations ratios and steady state fluorescence intensities at 367 nm excitation.

4.3.2. Study of antagonistic effects by microdialysis method

Infusion of ZM6 in the MBN elicited a significant extracellular elevation of Asp, Glu and taurine, with postponed peak response 40 min after the start of peptide infusion. Following a short excitatory response, excess extracellular Asp and Glu levels returned to the control value. In contrast, Aβ[1-42] infusion in the MBN elicited sustained elevation of excitatory amino acids, which was apparently present between 10 and 60 min following

dialysis of Aβ[1-42]. Aβ[1-42] induced extensive increases in Asp and Glu concentrations with an abrupt onset. 60 min after the start of Aβ[1-42] infusion in the MBN, the extracellular amino acid levels gradually decreased and returned to the baseline level after 100 min. ZM6 exerted a characteristic pharmacological action as it significantly decreased Aβ[1-42]-induced elevations of Asp concentration and eventually abolished Aβ[1-42]-induced enhanced Glu release. Pre-treatment with ZM6 did not influence the release profile of taurine, as peak taurine concentrations after either a combined ZM6 + Aβ[1-42] or single Aβ[1-42] administration did not differ significantly from each other. Reversed administration of Aβ[1-42] + ZM6 did not result in any noticeable antagonistic effect (Figure 3).

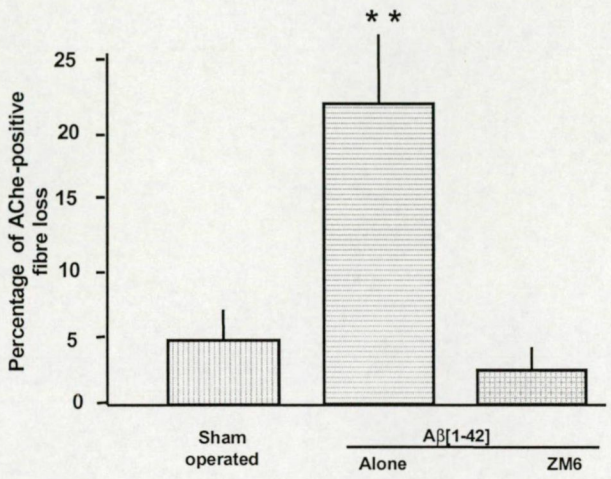


**Figure 3.** Loss of cholinergic (AChE-positive) projection fibers 14 days postsurgery as a consequence of Aβ[1-42] infusion into the MBN and the neuroprotective effects of the ZM6. Six sham-operated, six Aβ(1-42)-infused and four ZM6 + Aβ(1-42)- infused rats were used.

4.3.3. Behavioural tests

Assessment of spontaneous behaviours 24 h postlesion revealed a pronounced increase in the openfield latencies of Aβ[1-42]-infused animals, compared with other groups examined. In parallel with the changes of start latency, Aβ[1-42]-treated rats exhibited markedly decreased horizontal motor activities compared with both sham-control and ZM6 + Aβ[1-42]-treated rats, whereas rearing parameters did not change as a consequence of Aβ[1-42] infusion. Aβ[1-42] microdyalisis in the MBN resulted in sever disturbances of passive avoidance learning, which were indicated by significantly decreased post-shock latency periods of Aβ[1-42]-infused animals, whereas ZM6 attenuated the behavioural dysfunctions.

Infusion of Aβ[1-42] in the MBN elicited a significant loss AChE-positive projection fibres in the somatosensory cortex, compared with all other experimental groups investigated, which was effectively antagonised by ZM6 pre-treatment (Figure 4).

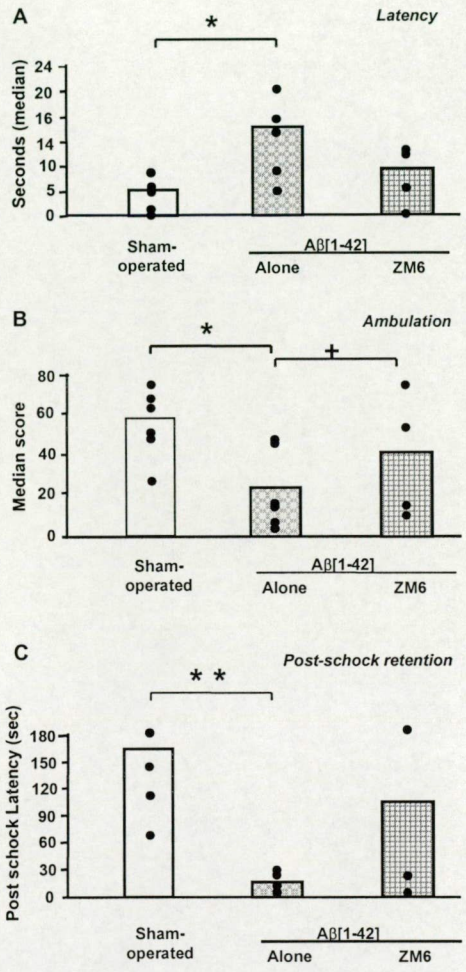


**Figure 4.** The effects of Aβ[1-42] on novelty-induced behaviours in the open-field 24 h post-surgery (A, B) or short-term memory in the postshock trial of a passive avoidance task (C).

4.3.4. Effect of Aβ[1-40] on transmembrane potential of cultured M26-1F cells measured by flowcytometry

Cultured M26-1F cells were labelled by passive diffusion on incubation with 2 μM bis-oxonol for 10 min at 25 °C. The cells were exposed to a 1 μM final concentration of Aβ[1-40] for 20 min. The effect of Aβ[1-40] peptide on the resting transmembrane potential was monitored by means of comparative cytofluorimetric studies. The flowcytometric

measurements demonstrated that the distribution of the individual cell-associated probe fluorescence was shifted to lower levels in cells treated with Aβ[1-40] for 20 min as compared with that of their untreated counterparts (Figure 5, left panel). A change in the fluorescence intensity in the same direction was caused by valinomycin, whereas gramicidin induced a shift to higher fluorescence intensities (Figure 5, right panel). Valinomycin is a $K^+$-specific ionophore known to induce hyperpolarisation [145], whereas gramicidin is a channel-forming $Na^+/K^+$-ionophore causing rapid collapse of the transmembrane potential [146].

Unfortunately, this method did not show adequate sensitivity to characterise and to compare peptides with supposed antagonist effects.

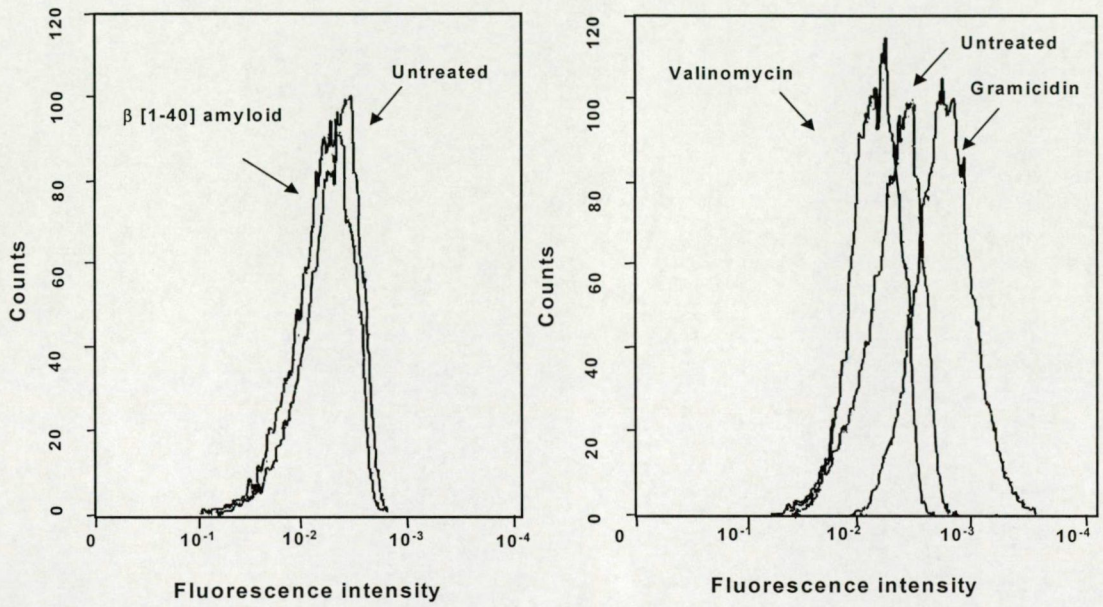


**Figure 5.** Representative flow-cytofluorimetric histograms of M26-1F cells with 1 μM Aβ[1-40] (left panel) and 1 μM valinomycin or gramicidin D (right panel).

### 4.4. Stability studies on Aβ[25-35] peptide

Some amyloid derived peptides show their toxic effects only after a long 'aging' period performed several days at pH 7.4 and 37 °C for cluster formation. Only the high aggregated, polymeric forms of Aβ peptides are neurotoxic; monomers show some neuromodulatory effects. "Aging" of Aβ peptides is a common method before *in vivo*

experiments [130, 131, 132]. *In vitro* investigations of Aβ neurotoxicity in cell and tissue cultures proceed for several hours or even days [133, 134] at pH 7.4, 37 °C. However, these long "aging" and incubation processes could result in degradation of Aβ peptides which contain Asn and Met in the peptide sequence. It is known that spontaneous cleavage occurs at the peptide bond adjacent to Asn [132,147,148,149].

Stability investigations were carried out under physiological conditions in the presence or absence of $Ca^{2+}$. Each experiment was repeated three times. The rate of degradation in the repeated experiments was similar. The possibility of biological degradation can be precluded because of the use of $NaN_3$ and sterilised water in sterile and sealed vials.

The investigations were first performed in distilled water and under physiological ion conditions at 37 °C in the presence of $Ca^{2+}$. It was found that, in distilled water at 37 °C Aβ[25-35] was stable without any signs of decomposition, even after 5 days (Figure 6).

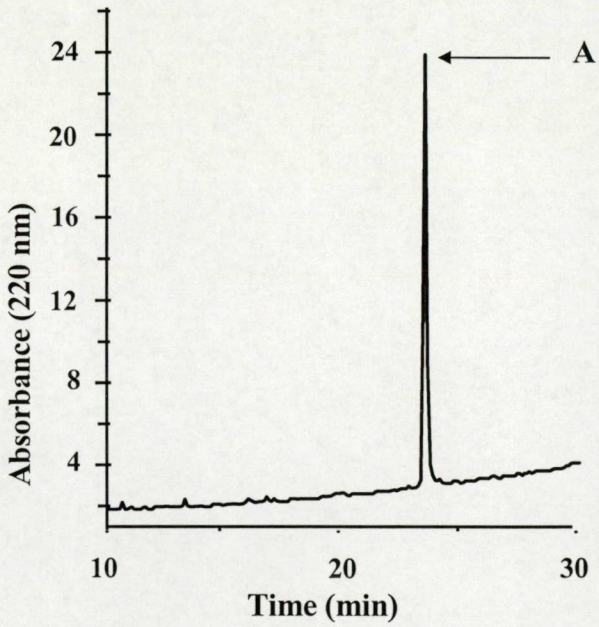


**Figure 6.** HPLC-chromatogram of Aβ[25-35] after a 5-day incubation in distilled water.

At physiological ion concentration and in buffer solution at pH 7.4, Aβ[25-35] was stable during the first 12 h at 37°C, but after 19 h a new peak appeared in the RP-HPLC chromatogram (Figure 7, left panel). After 5 days, more than 50% of the peptide was decomposed. (Figure 7, right panel).

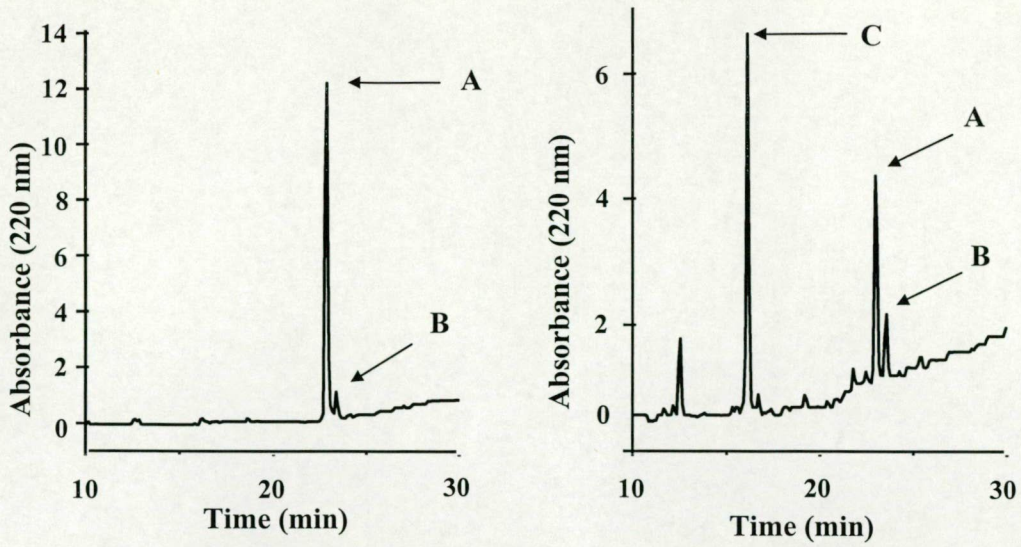


**Figure 7.** HPLC-chromatograms of Aβ[25-35] after 19 hours (left panel) and after 5 days (right panel) under physiological conditions. Aβ[25-35] peptide is denoted by letter A, Aβ[28-35] peptide by letter B and Aβ[28-35](Met-O) by letter C.

The compounds which represent peaks on the analytical HPLC were isolated and lyophilised, and their $R_t$ values were compared with those of the following synthetic fragments of Aβ[25-35]: Aβ[27-35], Aβ[28-35], Aβ[31-35] and Aβ[25-30]. These fragments were synthesised as reference standards for HPLC and Ms (Table 5). The presence of the peptide fragment Aβ[28-35] ($M_w$ = 800) and the absence of the fragments Aβ[27-35], Aβ[31-35] and Aβ[25-30] were demonstrated. Mass spectrometry revealed the $Ca^{2+}$ salts of the peptides Gly-Ser-Asn-OH and Gly-Ser-Asp-$NH_2$ (identical molecular weight: $M_w$ = 276), Aβ[28-35] and the oxidised form of Aβ[28-35] ($M_w$ =816).

At pH 7.0 only slight decomposition was observed. Experiments were also carried out at pH 8.0 in phosphate buffer, both in the presence and in the absence of $Ca^{2+}$. The results indicated that the degradation process accelerates at pH > 7.0 and in the presence of $Ca^{2+}$ (Figure 8).

| Fragments of Aβ | Mass spectra | |
|---|---|---|
| | Molecular mass | |
| | Calculated (average mass) | Formed |
| Aβ[25-35]-$NH_2$ | 1059.29 | 1059.0 |
| Aβ[28-35]-$NH_2$ | 801.06 | 800.0 |
| Aβ[27-35]-$NH_2$ | 915.16 | 915.0 |
| Aβ[25-30]-$NH_2$ | 531.57 | 531.0 |
| Aβ[25-30]-OH | 532.57 | 532.0 |
| Aβ[30-35]-$NH_2$ | 615.83 | 615.0 |
| Aβ[31-35]-$NH_2$ | 544.76 | 544.0 |
| Aβ[25-27]-$NH_2$ | 275.22 | 275.0 |

**Table 5.** Molecular mass values of Aβ fragments.

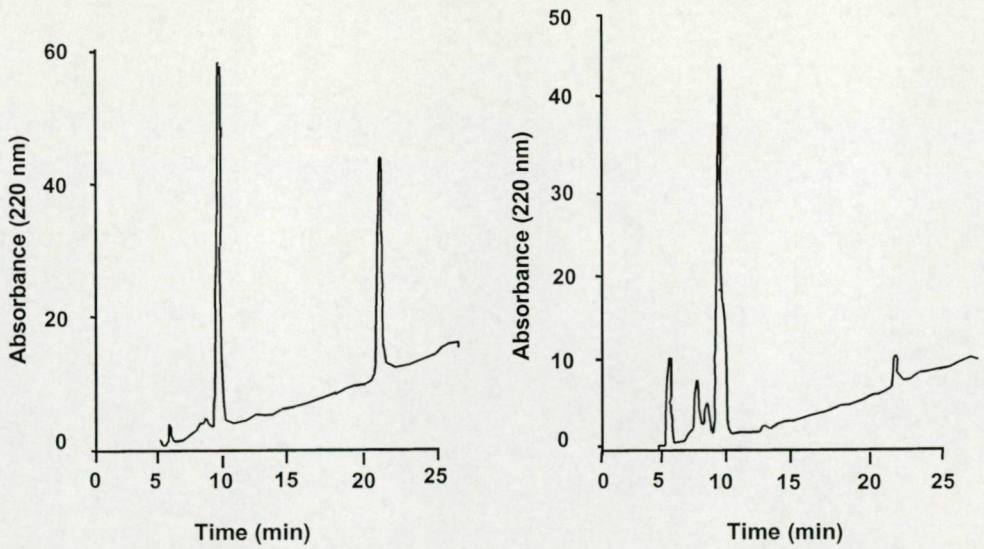


**Figure 8.** HPLC-chromatograms of Aβ[25-35] after incubation for 66 hours in a pH 8.0 buffer either in the absence (left panel) or presence of $Ca^{2+}$ (2 mM, right panel).

Stability investigations under argon atmosphere were performed in the absence of $Ca^{2+}$. The results suggest that the oxidation of Met can be avoided under these conditions. Degradation of the peptide chain occurs at only a very low rate (Figure 9).

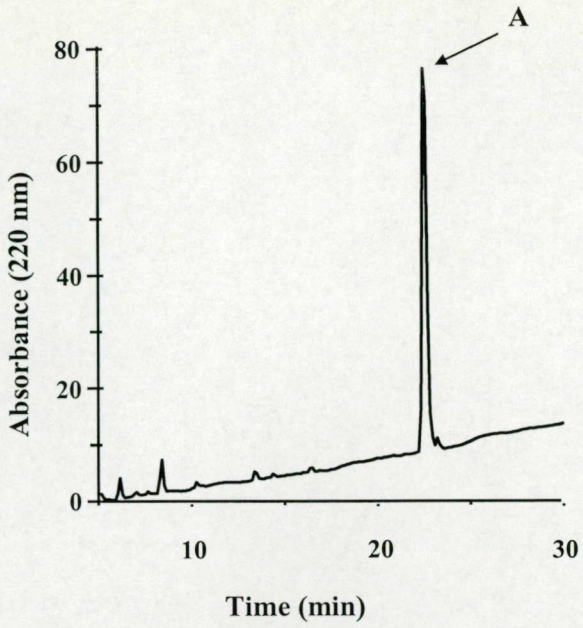


**Figure 9.** HPLC-chromatogram of Aβ[25-35] (denoted by letter A) after storage for 52 hours under an argon atmosphere at pH=8.0, in the absence of $Ca^{2+}$.

# 5. Discussion

## 5.1. Synthesis of Aβ peptides

Synthesis of a peptide with a well defined sequence of amino acids is a fairly complex process. Formation of the peptide bond through solid-phase peptide synthesis (SPPS) is the most commonly applied procedure. It is based on the stepwise addition of α-amino or side-chain protected amino acids to an insoluble polymeric support. The peptide can be cleaved as a peptide amide or acid, depending of linker to the resin. For N-α protection several blocking groups were recommended. The acid-sensitive Boc group, applied from the earliest days of SPPS, remained in a position of near-monopoly. Adaptation of the base-sensitive Fmoc group appears to be much more successful, especially in case of difficult peptide sequences. In addition, the repetitive deprotection steps of the Fmoc-chemistry do not require TFA, which can lead to alteration of the peptide bond and acid catalysed side-reactions. Moreover, anhydrous HF, the generally used cleaving agent in case of Boc-chemistry, is highly toxic and reactive, environmentally dangerous. In case of Fmoc-chemistry, cleavage of the peptide-resin

bond and simultaneous deprotection is carried out by TFA. However, Boc-chemistry is much more economical as Fmoc-chemistry.

In order to obtain high peptide purity, purification with HPLC is required. Purification of poor soluble peptides is a difficult problem. Moreover, the use of TFA as acidic ion-pairing agent can results peptides as TFA-salts which caused us difficulties by FT-IR spectroscopy measurements.

The Aβ sequence is a typical "difficult peptide", due to the strong interchain stacking, with β-sheet conformation [14,66,150,151,152,153,154,155]. This phenomenon also occurs on the resin and causes low reagent accessibility during synthesis, which accounts for the poor coupling efficiencies [156].

Fortunately, in some cases, especially for short but poorly soluble peptides a very precise and cautious synthesis resulted, before purification, peptides with purity more then 96% and made needless the purification of the peptide (e.g. ZM6). On the other hand, in case of peptides with difficult structures, manually synthesis must be preferred against an automatic peptide synthesiser in order to verify all amino acid couplings during the synthesis and according to necessity, to repeat the coupling.

### 5.2. Biological investigations

The main goal was to determine the minimum sequence with biological effects of the Aβ and to get peptides which might protect from the neurotoxic effect. It is noteworthy to mention the homology of the C-terminal five amino acids (IIGLM) of the Aβ[25-35] sequence with the C-terminal five amino acids (XXGLM, where X is an apolar amino acid) of tachykinins. The partially overlapping peptides were designed with special emphasis on this sequence. A series of modified peptide fragments were designed, synthesized, and investigated with various biological methods. The peptide with code *ZM6* showed the best antagonistic effect. To the best of our knowledge, ZM6 was the first Aβ analogue capable of preventing the Aβ[1-42]-induced long-term elevation in $[Ca^{2+}]_i$ [143]. Moreover, ZM6 was found to protect cholinergic MBN neurons against Aβ[1-42]-induced neuronal damage. The underlying mechanism of the neuroprotective action of ZM6 might entail ligand-like displacement of Aβ[1-42] binding from its hypothetical receptor [157]. Further we found that R-ZM6 peptide, the Arg analog of ZM6 and the KLVGF (KLV6) sequence proved to be

much more active antagonists then the mother compound. These lead compounds opened the way for designing effective Aβ antagonists, peptides and peptidomimetics.

On the other hand, Aβ peptides exert neurotoxic effects in aggregated form. Only the highly aggregated, polymeric form of Aβ peptides are neurotoxic; monomers show some neuromodulatory effects. Above a threshold concentration, Aβ undergoes highly ordered protein aggregation in aqueous solutions resulting e.g. in the formation of senile plaque in AD [30]. Following β- and γ-secretase-mediated catabolism of APP, Aβ, released in a monomeric form in the extracellular space, begins a self-aggregation process. Synthetic peptides even from different batches from the same laboratory may exhibit significant differences in neurotoxic activity, therefore it is necessary to determine what parameters influence the aggregation of Aβ peptides *in vitro* [158]. Also, our results demonstrated the importance of peptide sequence to the aggregation ability: *MOD–3* peptide, which has mostly hydrophilic amino acids in the sequence, shows a minimal tendency for aggregation. In contrary, the aggregation ability of *MOD-4* and Aβ[1-42] are similar [159].

### 5.3. Stability of Aβ peptides

Deamidation of Asn residues is a major nonenzymatic degradation pathway for many peptides and proteins: this intrinsic instability serves as "molecular clock". Our studies show that Aβ[25-35] is not stable under physiological conditions: the peptide chain is hydrolysed at $Asn^{27}$ resulting in isomer tripeptide fragments (Aβ[25-27] with Asn or isoAsn at the C-terminal end) and the octapeptide Aβ[28-35] [160]. The cleavage mechanism may involve formation of a succinimide ring as described for other peptides [147,161] (Figure 10).

The cleavage of the peptide chain is also affected by the presence of $Ca^{2+}$ ions: increased $Ca^{2+}$-comlexation facilitates degradation and peptide bond cleavage. It is interesting that this Asn-Lys sequence has not been described in the literature for being sensitive to hydrolysis under mild conditions. As Aβ[25-35] undergoes a relatively rapid decomposition at pH 7.4 if the buffer contains $Ca^{2+}$ ions, long incubation times and "aging" processes of Aβ peptides should be avoided. According to our results, the method of choice would be a relatively short (24 hours) incubation of Aβ[1-42] at pH 7.4 in aqueous solution, but without $Ca^{2+}$ ions and under argon atmosphere in order to avoid oxidation of $Met^{35}$ [130].

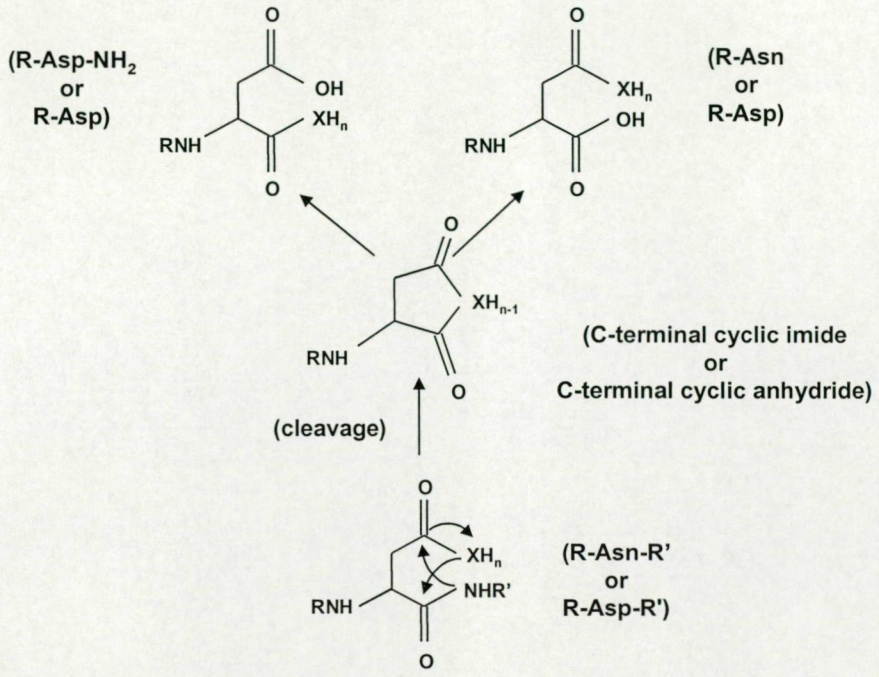


**Figure 10.** Proposed mechanism for cleavage at Asn (X=N, n=2) [161].

Our results support the necessity of stability studies of the peptides used in different biological experiments.

# 6. Summary and conclusions

The main findings of the PhD thesis are as follows:

1. Aβ[1-40], Aβ[1-42], two of its modified analogues, and several Aβ fragments were synthesized. The purified peptides were analytically characterized with HPLC, amino acid analysis and electrospray mass spectrometry. The synthesized peptides were used for stability studies, secondary-structure investigations and for biological experiments.

1/a. The solid phase synthesis of long Aβ peptides was optimized using Fmoc-chemistry and 10% anisol as an additive and deaggregating agent during the synthesis. This new method resulted in the best yields and purity known up to now.

2. Several *in vitro* and *in vivo* methods were applied (sometimes with some changes) for the measurement of the neurotoxicity of Aβ peptides as well as well the neuroprotective effect of short Aβ fragments and analogs (functional antagonists).

3. Fluorimetric studies using the $Ca^{2+}$ sensitive fluorescent probe Fura-2 AM demonstrated that rat astroglial cells treated with full-length Aβ peptides and its shorter fragments containing the sequence Aβ[31-35] and caused a long-term elevation of $[Ca^{2+}]_i$. The observed change can be considered as a long-term cell physiological action and can be used as a sensitive marker for testing for long-term activities of Aβ peptides.

   Peptide Pr-IIGL-amide (ZM6) was the first described peptide capable to reduce the βA[1-42]-induced long-term elevation in $[Ca^{2+}]_i$. This method can be adequate for testing several numbers of peptides with potentially antagonistic effects. Using microdialysis probes, by *in vivo* experiments were given further proofs of the neuroprotecting action of the ZM6 peptide.

3/a. We have designed and synthesized two new pentapeptides with very good neuroprotective effect, the KLVGF-amide and RIIGL-amide peptides. These compounds act as Aβ-antagonists also in very small amounts and serve as leads for further drug design.

4. Long aging of Aβ[25-35] under physiological conditions in the presence of $Ca^{2+}$ resulted in peptide bond cleavage between $Asn^{27}$ and $Lys^{28}$. The Asn-Lys sequence has not been described previously in the literature for being sensitive to hydrolysis under mild conditions. In distilled water at 37 °C, all the investigated Aβ peptides were stabile without any signs of decomposition even after aging for 7 day. For a long-term "aging" of peptides it can be recommended the incubation under argon atmosphere.

5. The aggregation ability of Aβ[1-42] and the *MOD-4* peptide, which does not contain the toxic fragment Aβ[31-35] and has mainly hydrophobic residues, are similar. In contrary, *MOD-3* peptide, which has mostly hydrophilic amino acids in the molecule, but contains the sequence of Aβ[31-35], shows a minimal tendency for aggregation.

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# 9. Annex

**Publications related to the subject of the Thesis**